Kidney & Blood Pressure Research

Purinoceptors and their Role in Pathophysiology

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Key Words

ATP, adenosine, cancer, dry eye, bladder instability, contraception, osteoporosis, pain, epilepsy, diabetes, thrombosis

The purinergic signalling field began over 30 years ago, but was not accepted until the 1990s after receptors for ATP were cloned and physiological roles established [see 1–4]. This brief article will summarise the current status of purinoceptor subtypes and focus on the increasing knowledge of the pathophysiology of purinergic signalling and its therapeutic potential.

Purinoceptor Subtypes

While the potent extracellular actions of purines were first recognised many years ago [5], it was not until 1978 that separate P1 receptors for adenosine and P2 receptors for ATP and ADP were postulated [6]. After transduction mechanisms for the actions of ATP were reported [7] and receptors for ATP were cloned and characterised [8–11], the currently accepted framework for the P2 receptor nomenclature was proposed in 1994 by Abbracchio and Burnstock [12] following the earlier subclassification into P2X and P2Y receptors based on pharmacological criteria [13]. Receptors for purines and pyrimidines consist of two families: P2X ionotropic ligand-gated ion channel receptors and P2Y metabotropic G protein-coupled receptors. Currently seven subtypes of P2X receptors are known and six subtypes of P2Y receptors, as well as four types of P1 receptors. Details of the distribution, molecular biology, pharmacological properties and physiological roles of the different receptor subtypes are available [see 14,15] (Table 1).

Pathophysiology and Therapeutic Potential

There are an increasing number of pathophysiological roles for purinoceptors emerging, some of which have therapeutic potential [see 16,17].

The *anticancer activity of adenine nucleotides* was first described by Rapaport [18]. Intraperitoneal injection into tumour-bearing mice resulted in significant anticancer activity against several fast-growing aggressive carcinomas [19–22].

In the *auditory system*, ATP, acting via P2Y receptors, depresses sound-evoked gross compound action potentials in the auditory nerve and the distortion product otoacoustic emission, the latter being a measure of the active process of the outer hair cells [see 23]. Both P2X and P2Y receptors have been identified in the vestibular system P2X splice variants are found on the endolymphatic surface of the cochlear endothelium, an area associated with sound transduction. It has been suggested that ATP may regulate fluid homeostasis, cochlear blood flow, hearing sensitivity and development, and thus may be useful in the treatment of Ménières disease, tinnitus, and sensorineural deafness.

In the *eye*, ATP, acting via both P2X and P2Y receptors, modulates retinal neurotransmission affecting retinal blood flow and intraocular pressure. The ATP analogue β,γ-methylene ATP ($\beta\gamma$-meATP) has greater efficacy in reducing intraocular pressure (40%) than muscarinic agonists like pilocarpine (25%) or (β-adrenoceptor blockers (30%) [24]. In the ocular mucosa, $P2Y_2$ receptor activation increases salt, water, and mucus secretion and thus represents a potential treatment for dry eye disease [25]. In the retinal pigmented layer, $P2Y_2$ receptor activation promotes fluid absorption and may be involved in retinal detachment.

Urinary bladder function is regulated by parasympathtic nerve stimulation, resulting in bladder contraction P2X receptors on the smooth muscle [26]. Detrusor malfunction results in urge urinary incontinence, a major health problem in the aging female population. Studies of $P2X_3$ knockout mice have shown bladder hyporeflexia as well as reduced pain [27,28]. This and other results have been reviewed recently in relation to potential treatment of interstitial cystitis, obstructive and neurogenic bladder [see 4].

Several findings suggest a potential role for purines in *contraception and fertility*. For example, in male rat genitalia, antibodies to $P2X_1$ and $P2X_2$ subunits show immunoreactivity in the membranes of the smooth muscle layer of the vas deferens [29]. In male $P2X_1$ receptor knockout mice, fertility is reduced by 90% without affecting copulatory performance. This is due to a decreased sperm count in the ejaculate due to a 60% reduction in the sensitivity of the vas deferens to sympathetic nerve stimulation [30]. $P2X_1$ and $P2X_2$ receptors have also been implicated in erectile function, especially in diabetes [31]. A recent study of the rat testis has shown involvement of several P2X receptor subtypes in spermatogenesis [32].

Purinergic receptors have a strong presence in *bone cells* [33–35]. P2X and P2Y receptors are present on osteoclasts with P2Y receptors only being present on osteoblasts. ATP, but not adenosine, stimulates the formation of osteoclasts and their resorptive actions *in vitro* [33] and can inhibit osteoblast-dependent bone formation. The bisphosphonate clodronate, which is used in the treatment of Paget's disease and tumour-induced osteolysis, may act via osteoclast P2 receptors [34]. Modulation of P2 receptor function may have potential in the treatment of osteoporosis, rheumatoid arthritis, periodontitis, and osteopenia. A recent study has shown that very low (nM) concentrations of ADP acting through $P2Y_1$ receptors turn on osteoclast activity [36].

ATP elicits *pain* responses via $P2X_3$ or $P2X_{2/3}$ receptors and ATP may contribute to the pain associated with causalgia, reflex sympathetic dystrophy, angina, migraine, visceral and cancer pain [see 37,38]. The nucleotide is also a key mediator of neurogenic inflammation via its actions on neutrophils, macrophages and monocytes, activation of which results in cytokine production and release [39]. For visceral pain, a purinergic mechanosensory transduction mechanism has been proposed [38] where distension of tubes, such as the ureter, gut, salivary and bile ducts, and sacs like the urinary and gall bladder causes ATP release from the lining epithelial cells to act on $P2X_3$ receptors located on the subepithelial sensory nerve plexus to relay nociceptive signals to the CNS.

Microinjection of ATP analogues into the *brain* prepiriform cortex induced generalized motor seizures similar to those seen

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Table 1: Characteristics of purine-mediated receptors

Receptor		Main Distribution	Agonists	Antagonists	Transduction Mechanisms
P1 (adenosine)	A_1	Brain, spinal cord, testis, heart autonomic nerve terminals	CCPA, CPA	DPCPX, CPX, XAC	G_i (1–3); ↓AMP
	A_{2A}	Brain, heart, lungs, spleen	CGS 21680	KF17837, SCH58261	G_S; ↑AMP
	A_{2B}	Large intestine, bladder	NECA	Enprofylline	G_S; ↑AMP
	A_3	Lung, liver, brain, testis, heart	DB-MECA, DBX RM	MRS1222, L-268,605	G_i (2,3), $G_{q/11}$; ↓AMP ↑IP_3
P2X	$P2X_1$	Smooth muscle, platelets, cerebellum, dorsal horn spinal neurones	$\alpha\beta$meATP = ATP = 2meSATP (rapid desensitization)	TNP-ATP, IP_5I, NF023	intrinsic cation channel (Ca^{2+} and Na^+)
	$P2X_2$	Smooth muscle, CNS, retina, chromaffin cells, autonomic and sensory ganglia	ATP ⩾ ATPγS ⩾ 2mSATP ≫ $\alpha\beta$meATP ($pH + Zn^{2+}$sensitive)	Suramin, PPADS	intrinsic ion channel (particularly Ca^{2+})
	$P2X_3$	Sensotory neurones, NTS, some sympathetic neurones	2mSATP ⩾ ATP ⩾ $\alpha\beta$meATP (rapid desensitization)	TNP-ATP, suramin, PPADS	intrinsic cation channel
	$P2X_4$	CNS, testis, colon	ATP ≫ $\alpha\beta$meATP	–	intrinsic ion channel (especially Ca^{2+})
	$P2X_5$	Proliferating cells in skin, gut, bladder, thymus, spinal cord	ATP ≫ $\alpha\beta$meATP	Suramin, PPADS	intrinsic ion channel
	$P2X_6$	CNS, motor neurones in spinal cord	(does not function as homomultimer)	–	intrinsic ion channel
	$P2X_7$	Apoptotic cells in immune system, pancreas, skin etc.	BzATP > ATP ⩾ 2meSATP ≫ $\alpha\beta$meATP	KN62, KN04 Coomassie brilliant blue	intrinsic cation channel and a large pore with prolonged activation
P2Y	$P2Y_1$	Epithelial and endothelial cells, platelets, immune cells, osteoclasts	2meSADP > 2meSATP = ADP > ATP	MRS2279, MRS2179	G_q/G_{11}; PLCβ activation
	$P2Y_2$	Immune cells, epithelial and endothelial cells, kidney tubules, osteoblasts	UTP = ATP	Suramin	G_q/G_{11} and possibly G_i; PLCβ activation
	$P2Y_4$	Endothelial cells	UTP ⩾ ATP	Reactive blue 2, PPADS	G_q/G_{11} and possibly G_i; PLCβ activation
	$P2Y_6$	Some epithelial cells, placenta, T-cells, thymus	UDP > UTP ≫ ATP	Reactive blue 2, PPADS, suramin	G_q/G_{11}; PLCβ activation
	$P2Y_{11}$	Spleen, intestine, granulocytes	ARC67085MX > BzATP ⩾ ATPγS > ATP	Suramin, Reactive blue 2 ARC67085MX, ARC69931MX	G_q/G_{11} and G_s; PLCβ activation G_i (2); inhibition of adenylate cyclase
	$P2Y_{12}$	Platelets	ADP		

with *N*-methyl-D-aspartate and bicuculline [40]. $P2X_2$, $P2X_4$ and $P2X_6$ receptors are expressed in the prepiriform cortex, suggesting that a P2X receptor antagonist may have potential as an antiepileptic [41]. P1 (A_{2A}) receptor antagonists are being explored for the treatment of Parkinson's disease [42]. ATP, in combination with growth factors, can act to stimulate astrocyte proliferation, contributing to the process of reactive astrogliosis, a hypertrophic/hyperplastic response that is associated with brain trauma, stroke/ischaemia, seizure and neurodegenerative disorders [43].

In stratified epithelium of *skin*, $P2X_5$ receptors are associated with proliferating and differentiating cells, while $P2X_7$ receptors label apoptotic cells [44]. $P2X_5$ and $P2X_7$ receptor agonists and antagonists may have potential in the treatment of psoriasis, scleroderma, basal cell carcinoma, and for restenosis following angioplasty.

In relation to *diabetes*, ATP stimulates pancreatic insulin release via a glucose-dependent, P2Y receptor-mediated mechanism [45] and also modulates insulin secretion by interactions with ATP-sensitive potassium channels in islet β-cells.

ADP produces *platelet aggregation* and three purinoceptor subtypes are present on platelets, $P2Y_1$, $P2X_1$ and the recently cloned $P2Y_{12}$ receptors [46]. An orally active $P2Y_{12}$ antagonist is currently entering phase I trials as an antithrombotic agent.

ATP was recognised early as a neurotransmitter in non-adrenergic, non-cholinergic inhibitory nerves in the *gut* [see 1]. Purinergic synaptic transmission is now also established in both myenteric and submucosa plexuses and abnormalities in *motility* are being explored as potential targets for purinergic agents [see 3].

For *cardiopulmonary function*, ATP is a mediator of vagal reflexes in the heart and lung [37]. In anaesthetized rats, P2X receptors have been implicated in evoking a Bezold-Jarisch response (hyperventilation, bradycardia, hypotension, apnea). ATP and UTP, acting via $P2Y_2$ receptors, stimulate chloride secretion in airway epithelium and mucin glycoprotein release from epithelial goblet cells [47], enhancing mucociliary clearance and reflecting a potential treatment for cystic fibrosis and chronic bronchitis [25]. ATP may also have a direct role in asthma via its actions on bronchial innervation.

Finally, there is a wide distribution of different P2 receptor subtypes in the *kidney*, both along the nephron and in the vascular system [see 48–52]. The potential of purine-related drugs for the treatment of abnormalities of kidney function is only beginning to be explored.

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Imaging of Renovascular Disease

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Introduction

Atherosclerotic nephropathie is the cause of renal failure in up to 25% of patients aged 60 years or older.The exact prevalence of atherosclerotic nephropathie, however, is not known and the diagnosis missed in many patients. Therefore, a non-invasive screening test for direct visualization of the renal arteries with a high accuracy and specificity is required in order to identify prospectively patients with a hemodynamic relevant renal-artery stenosis, who could benefit from a revascularization procedure. Any imaging modality used should provide reliable anatomical data on the renal arteries and be able to determine the degree of stenosis with sufficient confidence.

In the following sections the advances in noninvasive imaging of renal arteries disease are discussed and recent developments analyzed. The today concurring imaging modalities are Computed Tomography Angiography (CTA), Magnetic Resonance Angiography (MRA) and Doppler Ultrasonography. Intraarterial Digital Subtraction Angiography (DSA) as an invasive method has lost its role as a primary imaging modality in recent years, but remains the gold standard in patients with conflicting results in non-invasive tests and is still required prior to any surgical and percutaneous interventions [1–4].

Doppler Ultrasonography

Doppler Ultrasonography has been evaluated extensively for the detection of renal artery stenosis. The lack of standardized examination protocols and the wide difference in reported accuracy among different centers have, however, prevented an universal acceptance of this technique. Still, apart from the low cost of this technique, it is readily available and can be used for repeat studies to document disease progression or the outcome of any form of intervention. The use of microbubble echoenhancers in combination with harmonic Doppler imaging has lately been shown to improve the diagnostic confidence of the operator and reduces failure in renal artery visualization. The renal resistance-index (level of resistance to flow in the segmental arteries) seems to be an accurate predictor for the outcome in patients who underwent angioplasty or surgery with regard to renal function and improvement in blood pressure levels. Thus Doppler Ultrasonography can not only provide morphological but also functional information, which might allow to identify prospectively patients who will benefit from a surgical or percutaneous intervention [3,5–7].

Computed Tomography Angiography

CTA plays an important role in the evaluation and management of primary renovascular disease. Imaging of the renal arteries has been greatly improved with the introduction of spiral CT following injection of iodinated contrast material and two-dimensional multiplanar reconstruction. In cases of severe stenosis, however, discontinuity of the vessel can be produced on the reconstructed images due to partial volume averaging or the presence of severe calcifications. Using real-time interactive volume rendering and maximum-intensity projection images, renal artery stenosis can be detected with a sensitivity and specificity of about 95%. With the advent of multislice CT, past limitations of CTA has been overcome. The excellent spatial and temporal

resolution provided by this new technology allows imaging of vessels smaller than 1mm in an dominant-arterial phase. With near isotropic imaging fine vascular structures can be depicted and detection of segmental artery stenosis should become possible [4, 8–10].

Magnetic Resonance Angiography

In the last years MRA has become a clinical standard for detecting renal artery stenosis. State-of-the-art MRA is performed today as three-dimensional contrast enhanced angiography, using the T1 shortening effect of gadolinium chelates to provide the predominant signal in the images. High performance gradients and high field strength enable 3D volume acquisitions within a single breath-hold, thus minimizing respiratory artifacts. The reduced acquisition time allows for predominant arterial imaging, thus overcoming the problem of venous enhancement. Multiple studies have demonstrated the high accuracy of MRA for detecting renal artery stenosis, reporting sensitivities and specificities between 95% and 100%. The lacking exposure to ionizing radiation and its high accuracy makes it an useful tool when imaging renal arteries in prospective renal transplant donors. The large field of view allows reliable visualization of accessory arteries, thus facilitating surgical planning. It is also ideally suited for monitoring transplant recipients, since there is no need of exposure to nephrotoxic iodinated contrast agents [11–13].

Conclusion

In conclusion, state of the art CTA and MRA provide both highly accurate anatomical information on renal artery stenosis and can detect significant narrowing with a high degree of accuracy. Which method to employ will depend mainly on available scanning time, cost and local expertise. Ultrasound has the advantage of low cost and is readily available, however there remains considerable technical difficulties, especially the ability to produce reproducible measurements in different centers. Intraarterial Digital Subtraction Angiography should be reserved for patients with unsatisfactory or doubtful results in non-invasive test and for those patients, who will require therapeutic intervention.

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Medication and Revascularization for Renovascular Disease

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Renal artery stenosis (RAS), mostly due to atherosclerosis, can cause both renovascular hypertension, a form of hypertension reversible with renal revascularization, and renal insufficiency. Patients with atherosclerotic RAS also have a very high risk of myocardial infarction and stroke. In a Swedish survey of 164 patients with RAS of $\geqslant 50\%$, the risk ratio for cardiovascular mortality using the normal population of Sweden, matched for age, as a reference was 5.7 [1]. Of the 44 patients who died during the 7.1-year follow-up period, 33 died from cardiovascular diseases and only 2 progressed to end stage renal disease (ESRD). RAS should be treated with a view to prolonging survival or dialysis-free survival. Age and associated vascular disease, blood pressure (BP) and antihypertensive treatment score, renal function and kidney size should all be taken into account when making decisions concerning the treatment of individual patients.

Medication

Therapy using diuretics, beta blockers, angiotensin-converting enzyme inhibitors (ACEI), aspirin and statins reduces mortality due to coronary heart disease, congestive heart disease and stroke in high risk hypertensive patients, particularly in elderly patients and in those with diabetes mellitus or a previous cardiovascular event. Most patients with atherosclerotic RAS have hypertension and associated cardiovascular risk factors, and many have symptomatic atherosclerosis elsewhere. They should be provided with pharmacological treatment according to current recommendations and those who smoke should be advised to quit.

Special attention should be paid to ACEI, however, because they can induce renal dysfunction in patients with bilateral stenosis, stenosis in a solitary kidney, or severe nephrosclerosis [2]. Short-term treatment with ACEI is safe in patients with RAS provided plasma creatinine concentration is closely monitored, with ACEI treatment stopped if plasma creatinine increases by 20% or more [3]. ACEI is probably safe in the long term in subjects with low grade ($<60\%$) stenosis in whom plasma creatinine concentration does not change during the first month of treatment [3]. In addition to plasma creatinine concentration monitoring, kidney length should be determined yearly because individual kidney function may be reduced on the most stenotic side despite stable overall renal function and this may result in progressive unilateral kidney atrophy [4].

Revascularization

The results of renal revascularization have been first documented in retrospective reports in which BP improvement was overestimated due to the placebo effect and optimization of drug treatment.

In an overview of 10 published series reporting BP outcome following percutaneous transluminal renal angioplasty (PTRA), Ramsay and Waller [5] found cure rates of 50% in patients with fibromuscular dysplasia RAS but only 19% in those with atherosclerotic stenosis. Rimmer and Gennari reviewed the literature concerning revascularization of atherosclerotic RAS with progressive renal failure [6]. Following surgery, 55% of patients had improved renal function with a mortality rate of 6%; the figures for PTRA were 41% and 5%.

Three recent trials have compared PTRA with antihypertensive medication to medication alone in patients with atherosclerotic RAS. Webster *et al*. [7] randomly assigned 55 patients with unilateral or bilateral RAS to two groups: intervention or medication alone. Six-month BP changes were similar in patients undergoing intervention and those given medication alone. Plouin *et al*. [8] randomly assigned 49 patients with unilateral RAS to two groups: PTRA (with stenting if deemed necessary) or medication alone. The primary outcome measure was the mean change from baseline in 24-hour ambulatory BP at 6 months. The average reduction in BP was similar for the two groups but PTRA reduced by 60% the probability of having a treatment score of 2 or more at termination ($p < 0.001$). In the trial carried out by van Jaarsveld *et al*. [9], 106 patients with RAS were randomly assigned to two groups: PTRA (with stenting if deemed necessary) or medication alone. The mean reductions in BP were similar in the PTRA and medication groups but the final treatment score was significantly lower in patients who had undergone PTRA.

Overall, differences in final BP between patients treated by PTRA and by medication in these trials were minimal and only a minority of patients undergoing PTRA were able to discontinue medication. Nevertheless, the number of antihypertensive agents required to control BP was lower following PTRA than for medication alone. This is an advantage of PTRA over conservative treatment in patients with resistant hypertension.

The Decision to Revascularize

PTRA is usually safe and effective in patients with fibromuscular dysplasia RAS [5], who are mostly young female with recent hypertension and normal arteries outside renal vasculature. Conversely, increasing age is associated with an increase in the extension and severity of atherosclerosis, which in turn increases the incidence of puncture site and renal artery complications and of cholesterol embolism. Elderly patients with vascular disease and silent RAS with normal or near-normal BP and renal function should not be exposed to the complications of renal artery revascularization unless they develop heart failure. In hypertensive patients with RAS and normal or near normal renal function, hypertension can be controlled by drugs alone in most cases and there is no clear advantage of revascularization over medication plus careful monitoring. Revascularization is clearly justified only in patients with permanent hypertension and RAS due to fibromuscular dysplasia, in patients with resistant hypertension and atherosclerotic RAS and in those who need ACEI due to a history of heart failure or myocardial infarction. Patients with atherosclerotic RAS and mild renal failure have a much higher risk of dying from a stroke or myocardial infarction that of progressing to ESRD [1]. Patients with atherosclerotic RAS and moderate to severe renal failure have a high risk of both ESRD and cardiovascular death. They are therefore candidates for revascularization. Unfortunately, no randomized trial has been conducted in these subjects and retrospective reports suggest that they are at high risk of procedural complications [5]. Revascularization should probably be undertaken in ARAS patients with rapidly deteriorating renal function [2] or in whom plasma creatinine concentration has increased by more than 20% during ACEI treatment [3]. If the kidney is less than 8 cm long, there is little chance of BP improvement or kidney function recovery [2].

PTRA is the first-line method [10], surgical bypass being reserved for patients with associated aorto-iliac disease and cases with PTRA failure. Stent placement is required if there is an elastic recoil with a residual stenosis of 30% or more, which is much more frequent in ostial than in truncal stenosis. Therefore, most radiologists propose primary stent placement for stenosis within the aortic wall or within 10 mm of the aortic lumen.

Follow-up

In patients who have undergone revascularization and in those given medication alone, BP and plasma creatinine concentration should be measured every three months. Kidney size and renal artery patency should be assessed yearly, and probably once every six months in those given medication alone with high-grade ($\geq$60%) or bilateral stenosis [4]. Repeat angiography is required in cases with a secondary rise in BP or plasma creatinine concentration or if ultrasound assessment suggests restenosis or renal atrophy.

Conclusion

Most patients undergoing PTRA or surgery still need antihypertensive agents 6 or 12 months after the procedure. The reduction in treatment required by patients undergoing revascularization should therefore be weighed against the risks of complications and restenosis, particularly in cases with extended atherosclerosis and moderate to severe renal failure.

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Immunologie der Abstoßung diskordanter Xenotransplantate – Pathophysiologie und therapeutische Strategien

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Nach diskordanter Xenotransplantation Galα1-3Gal positiver Organe in Galα1-3Gal negative Empfänger kommt es zu einer Reihe immunologisch vermittelter Rejektionsphänomene im Xenotransplantat, welche als hyperakute Abstoßung (HAR), akut vaskuläre Abstoßung (AVR) und zelluläre Abstoßungsreaktion (CXR) bezeichnet werden können. Hierbei ist die Pathophysiologie der HAR diskordanter Xenotransplantate gut charakterisiert. Nach Transplantation Galα1-3Gal positiver Organe in Galα1-3Gal negative Empfänger kommt es durch Bindung präformierter Anti-Gal-Antikörper des Transplantatempfängers mit nachfolgender Komplementaktivierung zu einer sofortigen Endothelzellaktivierung im Transplantat. Diese Endothelzellaktivierung führt u.a. zu einer vermehrten Expression von Adhaesinen, zu einem Verlust der physiologischerweise anti-thrombogenen Eigenschaften des Endothels mit lokaler Thrombose und schließlich zur Aufhebung der strukturellen Integrität des Endothels mit folgender Zell-Lyse und generalisierter Thrombose. Per definitionem führt eine hyperakute Transplantatabstoßung in der diskordanten Kombination von beispielsweise Schwein auf Primat (Javaner-Affen, Rhesus-Affen, Paviane) innerhalb von 2 Std. zu einer kompletten und irreversiblen Thrombose des Transplantates. Das Auftreten einer hyperakuten Abstoßungsreaktion kann durch verschiedene the apeutische Interventionsstrategien verhindert werden: (1) Reduktion des Titers präformierter (sog. «natürlicher») Anti Gal-Antikörper durch beispielsweise Plasmapherese, Immunapherese, Donor-Organ-Perfusion. (2) Pharmakologische Inhibition des Komplementsystems (z.B. Cobra-Venom-Faktor, C1-Inhibitor, sCRI). (3) Verwendung Komplementregulator transgener Spenderorgane mit Expression von z.B. humanem CD55 (DAF) oder CD59. Insbesondere durch den Einsatz tran gener Spenderorgane stellt die HAR diskordanter Xenotransplantate in entsprechenden präklinischen Modellen heute kein relevantes Problem mehr dar.
Nach erfolgreicher Hemmung der HAR kommt es bei stabilen Herz- oder Nierentransplantaten trotz massiver konventioneller Immunsuppression derzeit regelhaft zu einer verzögerten Form der Xenotransplantatabstoßung der sog. AVR oder DXR («Delayed Xenograft Rejection»). Die AVR tritt in der Regel erstmals 4-8 Tage nach Transplantation auf und ist überwiegend humoral vermittelt. Bei manifester AVR werden in der Zirkulation der Transplantatempfänger häufig Titeranstiege für antiporcine Antikörper vorwiegend vom Isotyp IgM nachgewiesen. Entsprechend kann immunhistochemisch eine vermehrte Ablagerung von IgM-Antikörpern des Empfängers im Transplantat gezeigt werden. Mittels spezifischer IgM-Depletion kann in Großtiermodellen (Immunapherese) oder in Kleintiermodellen (Einsatz von monoklonalen Anti-IgM-Antiköpern) die AVR suffizient und zuverlässig verhindert werden. Einen anderen Therapieansatz zur Behandlung der AVR stellt die Inhibition der auf die Antikörperbindung folgenden klassischen Komplementaktivierung mittels Gabe von C1-Inhibitor oder sCRI dar.
Bisher sind erst wenige präklinische Modelle der diskordanten Herz- oder Nieren-Xenotransplantation mit Überlebenszeiten von mehr als 2 Wochen beschrieben worden. Erste Daten aus diesen Modellen zeigen, daß selbst nach suffizienter Behandlung einer AVR eine zellulär vermittelte Abstoßungsreaktion auftreten kann (CXR), welche durch multifokale zelluläre Infiltrate von überwiegend CD8-positiven T-Zellen, CD20-positiven B-Zellen, Makrophagen und NK-Zellen charakter-isiert ist. Die Bedeutung klassischer T-Zell- oder B-Zell-Immunsuppressiva zur Verhinderung einer zellulären CXR ist nicht geklärt.
Es ist denkbar, daß ein Langzeitüberleben diskordanter Xenotransplantate mit den derzeit realisierten transgenen Modifikationen der Spenderorgane auch in Kombination mit den o.g. immunsuppressiven bzw. immunmodulatorischen Protokollen nicht erreicht werden kann. Die Bearbeitung entsprechender Protokolle zur Induktion einer Xenotoleranz erscheint daher unverzichtbar. Die vielversprechendsten Ansätze zur Toleranzinduktion in der diskordanten Xenotransplantation sind hierbei derzeit wohl in non-myelo-ablativen Protokollen unter Einsatz von Spender-Knochenmarkstransplantationen zur Induktion eines Makrochimärismus zu sehen.

Newer Immunosuppressive Agents Used in Renal Transplantation

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Introduction

After transplantation of solid organs cellular immunological events directed against alloantigens lead to rejection and destruction of the graft if immunosuppressive agents are not applied. It is supposed that the immune system combats foreign antigens in a similar way as invading microorganisms or tumors where antigens presented as peptide fragments in association with self human leukocyte antigens (HLA) are targeted. However, in the transplant setting non-self HLA must be recognized by T cells. Thus, the number of mismatches of HLA class I (HLA-A, -B, -C) and class II (HLA-DP, -DQ, -DR) will determine the likelihood and strength of a rejection [1]. Antigen recognition leads to activation of T cells with consecutive amplification of immune responses but besides this anergy or apoptosis of lymphocytes can occur in the transplant setting [2]. Most immunosuppressive drugs used after transplantation interfere with T lymphocyte activation or closely associated cascades.
Ideally, effective immunosuppression would induce immunologic tolerance or anergy but current therapies are not very effective in this way. Because the balance between prevention of an acute rejection and overimmunosuppression is subtle newer immunosuppressive agents should be selective for alloantigen-driven processes and their effects reversible. The danger of over-immunosuppression can be minimized by the combined

application of different drugs directed againt distinct immune targets in concentrations as low as possible. Generally, immunosuppression is continuously administered as long as the transplant functions but T cell reactivity to alloantigens normally declines over the years. This process is roughly explained by adaptation processes between the host and the graft but can clearly lead to a declining demand of immunosuppressive therapy. This experience should be utilized in clinical practice and immunological test systems are needed which are suitable to indicate the lowest demand of immunosuppression without the danger of rejection.

Maintenance Immunosuppressive Therapy

After renal transplantation a triple drug immunosuppression is mostly applied at least for the first 3–6 months. It consists of glucocorticosteroids (GS), a calcineurin inhibitor (cyclosporin A or tacrolimus) and an antiproliferative substance such as azathioprine or mycophenolat mofetil (MMF).

GS have immunosuppressive, antiinflammatory and antiproliferative effects on various immune cells. They bind to a steroid receptor and the complex created enters the nucleus and binds to DNA sequences. GS interact with the transcription factors NF-κB and AP-1 which regulate numerous immune activation processes. In higher doses GS can evoke apoptosis of lymphocytes. The multiple side-effects of GS are well-known and cushingoid features of patients should lead to not too slow tapering or even ultimate withdrawal of steroids. Different trials evaluating the effect of GS withdrawal showed that this policy is practicable mainly in patients with serious metabolic side-effects induced by GS but that acute rejections and more often a slow decline of transplant function can occur. Thus, cessation of steroid therapy cannot be recommended for most transplant patients [3].

Cyclosporin A and tacrolimus inhibit calcineurin, a calcium-dependent serine phosphatase, which is centrally engaged in T cell activation. After activation via the T cell receptor (TCR) and opening of membrane calcium channels calcineurin is stimulated leading to dephosphorylation of different transcription factors. The "nuclear factors of activated T lymphocytes" (NFAT) are the key targets which migrate into the nucleus, bind to DNA sequences and interfere with cytokine gene promotors [4]. Cyclosporin binds to the immunophilin, cyclophilin, tacrolimus to the FK-binding protein 12 (FKBP12), and the formed complexes bind to calcineurin and block its phosphatase site. By NFAT deactivation most genes involved in T cell activation and proliferation (e.g. IL-2, IL-4, IFN-γ, CD40L) are blocked or down-regulated [5].

Cyclosporin and tacrolimus are the key columns of maintenance immunosuppressive therapy. With the introduction of cyclosporin A in the early 1980s a major breakthrough in the prevention of acute rejections was achieved. Side-effects of both drugs show similarities and differences. Both are nephrotoxic and can injure the tubulus system with consecutive hyper- or hypokalemia and hypomagnesemia. They cause hypertension at least partially by contracting preglomerular and other resistance arteries and exert neurotoxicity. Cyclosporin administration is more closely linked with hyperlipidemia and hirsutism, tacrolimus more with glucose intolerance and tremor.

The immunosuppressive capacity goes in parallel with the achieved plasma levels and short-term or middle-term transplant survival rates are similar between both the drugs. Tacrolimus is a potent drug to perform a rescue therapy in rejecting patients taking cyclosporin.

Azathioprine is an antimetabolite which is metabolized in the liver to 6-mercaptopurine and further converted to thioinosinic acid as the main active compound. It is incorporated as a purine into DNA and so acts as an antiproliferative agent with some selectivity for the hematopoietic and immune system. Embedded in a triple immunosuppressive therapy, azathioprine is typically given in a dose of 1.5 mg/kg per day. The main side effects are anemia, thrombocytopenia and most often leukopenia which is a frequent reason to discontinue or decrease the dose of the drug. Another serious adverse event is hepatotoxicity normally becoming evident by increasing cholestatic serum parameters.

MMF is a newer immunosuppressive drug used as a substitute for azathioprine as a part of a triple immunosuppression together with a calcineurin inhibitor and prednisolone. MMF is hydrolyzed in the liver by esterases to form the active compound mycophenolic acid (MPA). MPA impairs lymphocyte functions including proliferation by blocking guanosin biosynthesis via reversible inhibition of the enzyme inosine monophosphate dehydrogenase. MPA is further glucuronidized in the liver and mainly excreted with the urine and to a lesser degree via enterohepatic circulation in the bile. MMF is given in doses of 1–3 g bid. It is not nephrotoxic, bone marrow suppression may occur but less frequently as compared to azathioprine. However, gastrointestinal toxicity is not uncommon manifested by diarrhea, abdominal cramps and gastritis. Large multicenter trials comparing MMF with placebo or azathioprine in combination with cyclosporin and prednisone have confirmed efficacy in reducing acute rejection episodes and rates of steroid resistant rejections [6]. However, benefits for long-term renal transplant function and survival or as an effective therapy for ongoing chronic rejection are not approved so far.

Primary and maintenance immunosuppressive therapy currently applied in the Münster transplant center for kidney allograft recipients is summarized in Table 1.

New Immunosuppressive Drugs

Sirolimus (rapamycin)

Sirolimus is a macrolide antibiotic which is like tacrolimus produced by the fungus Streptomyces hygroscopicus. Like tacrolimus it binds to the immunophilin FK-binding protein (FKBP12) but without blocking calcineurin dependent IL-2 transcription. In contrast, rapamycin-FKBP12 complex binds to the kinase "target of rapamycin" (TOR) and prevents its activation. Consecutively, cell-cycle dependent signals and proteins are

Table 1: Primary immunosuppressive regimen currently applied in the Münster transplant center after renal transplantation

a. **Normal immunological risk** (e.g. first transplant, haploidentical living related donation)
cyclosporin A GS or MMF
MMF → withdrawal after 1 year
GS
b. **High immunological risk** (2.,3. transplant, high panel reactive antibodies, living unrelated donation)
tacrolimus
MMF or sirolimus
GS
c. **Old recipients with old grafts** (>65 years)
CD25 MAB
cyclosporin A
GS

inhibited leading to an arrest of the cell cycle of lymphocytes but also of non-immune cells. Because of the different mechanism of action as compared to calcineurin inhibitors there is a synergistic or additive effect together with cyclosporin or tacrolimus [7,8].
Similar to calcineurin inhibitors metabolism of sirolimus engages the cytochrom-P-450-3A4 pathway, but half-life is longer with 2.5 days. Thus, oral administration once a day is sufficient, normally in daily doses of 2 mg. Because of competitive interactions in drug uptake and metabolism, sirolimus is administered at least 4 hours after cyclosporin normally during noon. Sirolimus increases cyclosporin levels so that doses should be carefully adjusted. The plasma levels recommended for sirolimus are around 10 ng/ml but primarily depend on the concomitant immunosuppressive protocol [8].
Clinical trials have shown that sirolimus in combination with cyclosporin and glucocorticoids can reduce the risk of acute rejections substantially and may act cyclosporin-sparing. Frequent side effects are thrombocytopenia and mild myelosuppression. The induction of hyperlididemia and hypercholesterinemia which can be observed in more than 50% of patients is of major concern because many transplant patients are burdened with a high cardiovascular risk *per se*. The early peak of hyperlipidemia under sirolimus treatment is declining over the months post transplant. Since outbreaks of Pneumocystis carinii (PC) infection have been described in pilot studies, a parallel administration of PC prophylaxis is obligatory. Occurence of skin and mouth ulcers can be observed as well as disturbances of wound heeling post transplant which may be explained by the antiproliferative action on nonimmune cells such as fibroblasts.
Sirolimus was approved in the beginning of 2001 in Europe in combination with cyclosporin for the first 2–3 months post transplant. After that time span cyclosporin or sirolimus should be finished to avoid overimmunosuppression and increased nephrotoxicity. However, large well-controlled multicenter studies confirming the safety of a dual sirolimus/GS therapy without calcineurin inhibition in the first year post-transplant or over longer times in patients with normal or increased immunological risk are lacking so far. Some optimistic expectations are allowed that sirolimus as a non- or minor nephrotoxic drug can improve the long-term results of kidney transplant survival in certain patients [9,10].

Protein Immunosuppressives

Polyclonal antibodies such as ATG or ALG raised in rabbits or horse after immunization with lymphoid cell lines, thymocytes or lymphocytes are well-established in renal transplantation and used as a part of an induction therapy or more frequently for treatment of steroid-resistent acute rejections.
Murine monoclonal antibodies (MAB) directed against the CD3 complex (OKT3) which block T cell triggering via TCR and finally lead to T cell depletion by apoptosis have a more specific target but are known for drastic side-effects. Especially after the first doses OKT3 can induce a cytokine release syndrome with hypotension, pulmonary edema due to capillary leak, headache, chills and fever. The patient can form neutralizing antibodies against the mouse protein which may lead to ineffectiveness.
Two new monoclonal antibodies directed against CD25, the α-chain of the IL-2 receptor (IL-2R), have been produced, tested in clinical studies and approved: the fully humanized MAB, daclizumab, and the chimeric MAB, basiliximab. The chimeric antibody bears the mouse protein part in the variable IgG region, whereas the humanized MAB possesses also human sequences within the variable region and merely residual antigen binding sites from the mouse. With respect to immunogenicity no differences exist between both MAB, however, basiliximab has a higher receptor affinity and a markedly shorter half-life. Since CD25 is not expressed by resting T cells and restricted to activated lymphocytes these MAB mean a first approach to specific allograft-restricted immunosuppression.
Basiliximab is administered at day 0, that means during or immediately before transplantation, and at day 4 post transplant with 20 mg each, daclizumab at a dose of 1 mg/kg body weight at an interval of 2 weeks 5 times post transplant. These anti-CD25 MAB are approved for acute rejection prophylaxis but not for treatment of acute rejections and are combined with dual or triple immunosuppression comprising a calcineurin inhibitor and glucocorticosteroids. It could be shown in controlled studies that the frequency of acute rejections could be reduced by 30%, with no or little side effects [11,12].
Other MAB and immunoglobulin fusion proteins are under development which block the CD28 costimulatory pathway or CD40 ligand, an essential molecule of activated T cells which is engaged in B cell stimulation and immunoglobulin class switching.

Other Immunosuppressives

Several new drugs with immunosuppressive potential are under development or in phase II and III clinical studies. 15-deoxyspergualin (DSG) is an antibiotic-like immunosuppressive isolated from cultures of Bacillus laterosporus and can inhibit lymphocyte proliferation and cytokine production by different modes of action. Clinical trials in North America have been stopped because of side effects, but in Europe a multicenter study for treatment of patients with ANCA-positive vasculitis is performed just now.
FTY720 is a fungus metabolite which is modified synthetically and shows synergistic effects with cyclosporin. Probably, it has novel effects on lymphocyte recirculation and homing by interfering with G protein coupled lymphocyte receptors [13].
Taken together, new immunosuppressive agents suitable and applicable for kidney or other solid organ transplantions are under development or newly approved and tested in clinical practice. The right composition of the immunosuppressive regimen is essential for the long-term fate of our patients. Pharmacodynamic and immunological tests are needed to determine the achieved grade of T and B cell but also monocyte/macrophage deactivation of transplant recipients in order to aid right dosing of drugs. New substances might help to prevent acute rejections safely, avoid to paralyse the immune system too rigorously and, probably, help to induce tolerance in the future.

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Extracellular Adenine Nucleotide Metabolism

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In the purinergic signalling field, the metabolism of extracellular nucleotides has several functions: i) the degradation of the nucleotides and thus terminating their signalling roles via membrane-bound nucleotide receptors (purinergic receptors P2 and P1); ii) a switch in the signal direction by the generation of other signalling molecules, e.g. the hydrolysis of ATP, which acts on P2 receptors, down to adenosine which then activates the P1 purinergic cascade. Thus, only further metabolism of adenosine to inactive ligands, or the uptake of adenosine by nucleoside transporters, ultimately terminates the signalling function of nucleotides in the extracellular space.

It has been demonstrated that at least two functional classes of nucleotides are found in the extracellular compartment: a) mononucleotides, such as ATP and ADP with half-lives of some minutes, b) "long-living" nucleotides such as diadenosine polyphosphates (ApnA; $n = 2–6$) which may exhibit their function as uncleaved molecules, but may also act indirectly as prodrugs via enzymatic liberation of mononucleotides such as ATP and ADP.

The metabolism of nucleotides in the blood has been studied by fractionating the plasma and purifying some main hydrolytic activities. The biochemical analyses revealed several so-called phosphdiesterases with broad cleavage specificities so that mononucleotides as well as dinucleotides are degraded in the blood plasma, revealing discrete differences in their half-lives.

The kinetics, specificities and possible functions of membrane-bound ecto-nucleotidases has been shown with many different cell systems (erythrocytes, white blood cells, endothelial cells, mesangial cells, dendritic cells). On many cell types, the whole cascade of ecto-nucleotide hydrolases is expressed, starting with phosphodiesterases cleaving dinucleotides, followed by ecto-ATPase, ectoADPase and finally ectoAMPase (5′-nucleotidase), showing different activities and expression patterns. An important finding is that the presence of other, sometimes competitive nucleotides may have dramatic influence on the metabolism of the substrate nucleotide, i.e. the half-life of the substrate nucleotide could be lengthened.

Among the ectoenzymes expressed in many cells there are, beside the hydrolases listed above, also nucleotide phosphotransferases such as adenylate kinase. For ecto-adenylate kinase as well as for a novel ecto-nucleoside diphosphate kinase, we have established coupled luminescence assays for biochemical and functional characterization.

Of physiological significance might be the finding that the various enzymes of the hydrolytic cascade as well as the phosphotransferases are compartmentalized in some tissues, i.e. different cells or cell layers in a tissue have different expression pattern of their ectoenzymes. Moreover, adaptive response of the expression pattern of ecto-nucleotide metabolizing enzymes have been found in model systems, e.g. during the inflammatory response of macrophages.

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Puringeric Receptors in the Mammalian Nephron

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P2 receptors are expressed in nearly all mammalian tissues. A myriad of different cellular functions have been shown to be regulated or modulated by extracellular nucleotides. Thus the field of P2 receptor research continues to grow tremendously and over the past decade has touched on nearly all subspecialties of basic life science including numerous clinical fields of research. Mammalian P2 receptors, activated by a number of different nucleotides like ATP, UTP, ADP or UDP, are subdivided into metabotropic (G-protein coupled) P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$) and ionotropic (non-selective Ca^{2+} permeable cation channel) P2X ($P2X_{1–7}$) receptors [1]. All P2Y receptor have been shown to link to an $Ins P_3$-mediated release of Ca^{2+} from internal stores [1]. Only very recently the long searched second P2Y receptor in thrombocytes could be identified ($P2Y_{12}$) [2]. The nephron has also been shown to be a rich source for the expression of P2 receptors. Here, I shall try to give a comprehensive overview of P2 receptor expression and their functional implications in the mammalian nephron.

It continuous to remain a difficult task to unequivocally demonstrate which of the different P2 receptors is responsible for the observed effects. Lack of truly specific agonists or antagonists, the often multiple expression of different P2 receptors in the same tissue and the extracellular metabolization of the agonist are hindrances in our undertakings. Thus often a P2 receptors is identified, however lacking detailed sub-classification.

Expression of P2 Receptors in Cultured Renal Epithelial Cells

Numerous studies have identified several P2 receptors in cultured cell lines derived from proximal to distal tubular origin. With functional and molecular techniques either a $P2Y_1$, $P2Y_2$ or $P2Y_6$ receptor were identified [3,4]. No evidence for other P2Y receptors has been presented. In addition RT-PCR data suggests the expression of P2X receptors with the shortcoming of convincing functional data [3]. In polarized renal epithelial cells grown on filter supports the expression of $P2Y_2$ receptors was shown also in the luminal membrane [4,5].

Expression of P2 Receptors in Intact Nephron Segments

Studies investigating the expression of P2 receptors in the intact nephron of mouse, rat or rabbit are still limited. They suggest however, that P2 receptors are expressed along the entire nephron [6]. Most studies used non-perfused isolated nephron segments and therefore demonstrate P2 receptor expression in the basolateral membrane. In the Proximal tubulus $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptor expression was shown [6,7,8]. Further along $P2Y_2$ and maybe $P2Y_4$ receptor expression is suggested in the loop of Henle [6,9]. Most data have been generated in distal nephron segments. Here the expression of $P2Y_1$ and $P2Y_2$ receptors has been reported [7,10]. An intriguing finding suggests that the expression of P2Y receptors increases along the corticomedullary axis of the distal nephron [7]. Of notice is the fact that also in renal epithelial cells different P2Y receptors subtypes often appear to be expressed in the same cell type [3]. This coexpression of multiple P2 receptors is not yet understood.

Luminal P2 Receptors in the Intact Nephron

P2 receptors have the unique phenomenology of being most frequently expressed also in luminal membranes of nearly all transporting epithelia. Cell culture work in the mammalian nephron also suggested this [5,11]. Recently we were able to demonstrate luminal $P2Y_2$ receptor expression in principal cells of mouse cortical collecting duct [12]. Subsequently we could demonstrate that the activation of this $P2Y_2$ receptor inhibits electrogenic Na^+ transport [13].

Functional Roles of P2 Receptor Activation in the Nephron

As implicated above, the inventory of P2 receptors and their site of expression in the nephron is likely to be incomplete. The critical task will be to investigate the physiological role of P2 receptors in the nephron. A number of concepts for the physiological significance of extracellular nucleotides in renal tubular function have evolved. *1. Ischemic protection hypothesis*: A few important studies using intact collecting ducts show that $P2Y_2$ receptor activation inhibits the main transport processes of the principal cell: ROMK-mediated K^+ secretion [14], ENaC-mediated electrogenic Na^+ absorption [13] and AQP2-mediated H_2O transport [15,16]. The sequence of events could be the following: ATP has been shown to be released from epithelial cells by mechanical stimulation or cellular swelling [17,18]. In an event of ischemia epithelial cells will suffer energy-depletion and swelling. This in turn may trigger "regulated" ATP release and auto- or paracrine P2Y receptor stimulation. Thus ATP may autoinhibit energy-consuming transport processes. *2. Cellular growth and regeneration*: Extracellular ATP has been shown to be a potent stimulator of cellular growth [19]. Again ischemia or any other damaging threat could be the initial event. ATP would inevitably be released and could stimulate neighboring cells to proliferate in an effort to close the epithelial barrier defect. *3. Short-term regulation of Na^+ transport*: The state of Na^+ absorption is usually set by the long term action of aldosterone or ADH. ATP may serve as short term Na^+ transport regulator. One may speculate that ATP is involved in the well described phenomenon of Na^+ transport feed-back regulation. Whenever basolateral Na^+ extrusion via the Na^+/K^+ ATPase is compromised, cellular swelling would occur unless luminal Na^+ entry is reduced. Cell swelling-mediated ATP extrusion in turn could, via basolateral and luminal P2Y receptors, inhibit luminal Na^+ entry and thus help to reestablish the balanced luminal Na^+ uptake and basolateral Na^+ extrusion. *4. ATP and cell volume regulation:* Numerous studies using renal epithelial cell cultures have demonstrated activation of Ca^{2+}-mediated of Cl^- channels [3,4,5]. In a concept derived from bile duct epithelial cells, it was shown that ATP released after cellular swelling may serve to activate regulatory volume decrease (RVD) [20]. This concept may be especially attractive for the nephron, a site with a high demand for efficient cellular volume regulation. In intact nephron segments however, Ca^{2+}-activated Cl^- channels could not be identified. It thus appears unlikely that KCl extrusion-mediated cell volume reduction truly occurs in the intact nephron. We believe that many cultures cells artificially express Ca^{2+}-activated Cl^- channels which will not be found in the intact tissue of reference.

Conclusion

P2Y receptors are widely expressed along the entire nephron and most evidence indicates their involvement in the regulation of solute and water transport. In similarity to many other epithelia the widespread expression of receptors in the luminal membrane holds also true for the distal nephron. Integrated functional concept for P2 receptors are still evolving and require rigorous testing.

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Vascular Effects of Extracellular Nucleotides

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Abstract

The extracellular nucleotides ATP, ADP, UTP and UDP are important for vascular regulation. They stimulate vasoconstriction, vasodilatation by release of prostaglandins, NO and EDHF, are involved in shear stress, are growth factors for vascular smooth muscle cells, stimulate platelet aggregation, tPA and PAI-1 release. The effects are mediated by P2 receptors with at least 13 subtypes. The physiological effect is dependent on the release of extracellular nucleotides, the degradation by ecto-nucleotides, the type of P2 receptors expressed on the cells, their desensitization rates and their second messengers. P2 receptors have highly specific organ distribution and they can be rapidly up or down regulated. These mechanisms constitute an "extracellular nucleotide system" which may be of similar importance as the sympathetic and renin-angiotensin-aldosterone systems in cardiovascular regulation and pathophysiology.

Key Words

Vascular, smooth muscle cell, endothelial cell, P2Y receptor, P2X receptor

Introduction

The potent cardiovascular effects of extracellular nucleotides have been known for many years [1]. P2 receptors mediate actions of the extracellular nucleotides ATP, ADP, UTP and UDP regulating several physiological responses including cardiac function, vascular tone, vascular smooth muscle cell (VSMC) proliferation, platelet aggregation and the release of endothelial factors. The P2 receptors are divided into two classes on the basis of their signal transmission mechanisms and their characteristic molecular structures, the ligand-gated intrinsic ion channels named P2X receptors and the G-protein-coupled P2Y receptor subtypes [2]. So far, the P2Y family is composed of six cloned and functionally defined subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$). The P2X family is composed of seven cloned subtypes ($P2X_1$–$P2X_7$) [3].

Evidence has been presented for a number of different local sources of purine nucleotides: ATP has been shown to be released as a cotransmittor with noradrenaline from perivascular sympathetic nerves and as a transmitter from purinergic and sensory nerves. Furthermore, ATP and ADP are components of blood borne elements, such as platelets, inflammatory cells and erythrocytes, and can also be released from endothelial (EC) and smooth muscle cells during hypoxia and shear stress [4]. Less is known about release of pyrimidines but UTP (which also will be degraded to UDP) can be released from both platelets and endothelial cells under physiologically relevant stimuli. In general, UTP is released in a ratio of 1 : 5 compared to ATP [5].

Vascular Contraction

Vasodilatation and vasoconstriction have been demonstrated in blood vessels from most regions of the body [3]. The first evidence of the presence of P2 receptor subtypes suggested P2X receptors on vascular SMC and P2Y receptors on EC [2]. This has been revised later as seven subtypes of the P2X and six of the P2Y receptor has been cloned. The P2X receptor was defined by Kennedy and Burnstock as sensitive and rapidly desensitized by $\alpha\beta$-MeATP, which fits the cloned $P2X_1$ receptor [2]. In rat blood vessels immunohistochemistry has shown this to be the dominating subtype in SMC [6]. However, contractile effects of UTP suggested that also P2Y receptors were present on VSMC's and mediated contraction [7]. However, due to contamination, degradation and interconversion it has been difficult to discriminate between the effects of UTP and UDP. Using the stable pyrimidines UTPγS and UDPβS it has been possible to show that contractile effects are mediated by both UTP sensitive $P2Y_{2/4}$ and UDP sensitive $P2Y_6$ receptors [8]. Furthermore, the stable pyrimidines are powerful vasoconstrictors and up to

1000-fold more potent than the endogenous ligands emphasizing the importance of ectonucleotidases. It has been difficult to prove the importance of other contractile P2X receptors besides $P2X_1$ due to the lack of specific agonists and antagonists. However, $P2X_7$ may be involved in possible contraction and cell lysis in human saphenous veins and thereby cause varicose veins [9].

Vasodilatation by Actions on Endothelial Cells

Extracellular nucleotides have several important effects mediated by activation of endothelial cells. Vasodilatation and decreased blood pressure by release of prostaglandins and nitric oxide (NO) has been demonstrated in several studies [3]. Evidence also suggests the existence of endothelium derived hyperpolarizing factor (EDHF), which relaxes VSMC's by activation of potassium channels with subsequent hyperpolarisation [10]. The chemical structure is not known but it is defined as released from endothelium, not blocked by inhibitors of NO or prostaglandins, inhibited by a combination of the potassium channel inhibitors charybdotoxin plus apamin and causing hyperpolarisation [11]. All these criteria has been fulfilled for P2 receptor mediated vasodilatation [12,13]. This was recently confirmed *in vivo*, in man where both UTP and ATP reduced forearm vascular resistance in a prostaglandin and NO independent way [14]. Since UTP is degraded to uridine, adenosine cannot explain the effects. EDHF has been shown to be more important than NO in blood vessels of smaller diameter [10]. These resistance vessels play a major role for the resistance measured during *in vivo* infusions. In the human forearm NO and EDHF inhibitors had no effect, indicating an important role for EDHF in P2 receptor mediated vasodilatation in man [14].

An interesting interplay between EDHF and $P2X_1$ receptors has been found, were $P2X_1$ receptors by their membrane depolarising effect specifically attenuates the hyperpolarising effects of EDHF [13,15]. Thus, ATP dilatations in vessels with a high proportion of EDHF mediated dilatations are attenuated by simultaneous $P2X_1$ activation on the VSMC, while UTP and ADP mediated dilatations are preserved. A strong tendency for more potent vasodilatations to UTP compared to ATP in man support this [14].

The prototypical endothelium receptor since the definition of the P2Y and P2X receptor subclasses is the $P2Y_1$ receptor and still even after cloning of six different P2Y receptors it seems to be of main importance in most vascular beds. UTP sensitive $P2Y_{2/4}$ receptors are also important, but UDP-mediated vasodilatation has not been described so far [3]. This was confirmed with stable and selective UDP agonist UDPβS that had no vasodilatory effects indicating that $P2Y_6$ receptors are absent on the endothelium [8].

The $P2X_1$ receptor is not expressed on the endothelium and the evidence for other P2X receptors have been scarce. However, the $P2X_4$ receptor has recently been shown to be expressed with high mRNA levels in endothelium (with low levels for the other P2X receptor subtypes) [16]. Using antisense oligonucleotides the $P2X_4$ receptor was shown to be important for shear stress dependent Ca^{2+}-influx via an ATP dependent mechanism [17]. This indicates that ATP and P2 receptors may be of importance for shear stress which is in agreement with the well established release of ATP from endothelial cells during shear stress [18].

Renal Circulation

Similar to other vascular beds the effects of extracellular nucleotides in the renal circulation are a balance between the contractile effects on the VSMC and the dilatory effects mediated by NO-release from the endothelium by $P2Y_1$ receptors. $P2X_1$ receptors are dominant in afferent but mainly absent in the efferent arterioles [19,20]. In the afferent arterioles the $P2Y_2$ receptor have vasoconstrictor rather than vasodilator effects [20]. The result is that extracellular nucleotides selectively influence preglomerular resistance without having an effect on postglomerular tone.

Extracellular Nucleotides as Growth Factors

Extracellular ATP is a potent growth factor for VSMC by activation of P2Y but not the ion channel coupled $P2X_1$ receptor [21–23]. This has been further expanded to encompass UTP acting on $P2Y_{2/4}$ receptors [22,24], 2-MeSADP acting on $P2Y_1$ and UDP acting on $P2Y_6$ receptors [25,26]. ATP is synergistic with polypeptide growth factors (PDGF, bFGF) and insulin [21, 22]. The signal transduction is mediated via Gq-proteins, phospholipase C and D, diacyl glycerol, protein kinase C, ERK, PI3K, MEK and MAPK [27,28]. Recently, the Rho-pathway has been implicated for several of the P2Y receptors [29]. Several immediate early genes are activated and depending on cell type and agonist the cell is taken through different phases of the cell cycle [23,30]. Sometimes, as for UDP acting on $P2Y_6$ receptors the progression is stimulated to both G_1 and S phase as measured with flow cytometry, i.e. through the whole cell cycle which was also confirmed by an increase in cell number [26].

The growth stimulatory effects of P2 receptors may be of importance in atherosclerosis, neointima development, chronic vascular rejection after transplantation and pulmonary hypertension. In these processes interaction between the VSMC and the matrix is important. It is therefore fascinating that the human $P2Y_2$ receptor contains an integrin-binding domain (RGD) in its first extracellular loop and that interaction with integrins influence $P2Y_2$ receptor mediated activation of G-proteins [31].

Human VSMC

Mitogenic effects of ATP on human VSMC has been demonstrated in cells from subcutaneous arteries and aorta were ATP acted in synergy with other sympathetic cotransmitters and polypeptide growth factors [32,28]. Since then we have cultured human VSMC from internal mammary, omental, cerebral and coronary arteries and also veins from the same tissues and saphenous veins. We have encountered several problems. First, the cells seem to have lost their ability to react via their G-protein coupled receptors. Despite trying angiotensin II, noradrenaline (and several specific agonists), 5-HT, endothelin, thromboxane analogues and of course multiple P2-receptor agonists we did not get (or only serendipitous) activation of the cells as measured by FURA-2, increase in cell number, cell cycle progression, total protein or ^{3}H-leucine-incorporation. The problems remained even when we used the first passages or tried every obtainable culture medium, different serum or serum free conditions. The second problem was the ^{3}H-thymidine incorporation assay. Here adenosine containing substances (ATP, ADP, AMP, adenosine, AP_4A, AP_5A, AP_6A) caused an increase in ^{3}H-thymidine incorporation of similar potency without matching any known receptor profile. Stable specific adenosine receptor agonists had no effect. Furthermore, it could not be confirmed by any other assay of cell growth (increase in cell number, cell cycle progression, total protein or ^{3}H-leucine-incorporation). A stimulatory effect of ATP on ^{3}H-thymidine incorporation has also been found in cells from mammary artery and saphenous vein

[33]. Furthermore, they found an inhibitory effect of UTP on ^{3}H-thymidine incorporation. We have also found similar effects of UTP but in our experiments UDP, UMP and uridine had the same inhibitory effect. Even more important, we could not confirm this inhibitory effect neither by a decrease in cell number, total protein, ^{3}H-leucine incorporation, inhibition of cell cycle progression by flow cytometry or in an increase of cAMP (which could have been a possible pathway). Therefore, we believe that adenosine-containing substances non-specifically stimulates incorporation of thymidine into cultured human VSMC and probably also endothelial cells [34], without any real growth stimulatory effects. We tested this by adding ^{3}H-thymidine during the first 2 hours of agonist stimulation without having it present during the DNA-synthesis phase (14–20 hours after stimulation) and the increased incorporation was of similar magnitude as if it was present during the latter phase. This was not the case in rat VSMC were ^{3}H-thymidine incorporation is accompanied by an increase in the other methods to study cell growth and ^{3}H-thymidine incorporation mainly occurs in the DNA-synthesis phase. It is possible that there is a cell membrane transporter protein for thymidine and other nucleosides that is stimulated by adenosine and inhibited by uridine containing substances in a non-P2 receptor dependent way. These problems calls for studies on intact blood vessels devoid of the artifacts of cultured human VSMC.

Regulation of P2 Receptor Expression

The physiological effect is dependent on the type and numbers of P2 receptors expressed on the cells. It turns out that P2 receptors have highly specific organ distribution and also that they can be rapidly up or down regulated. Pacaud and coworkers found that the Ca^{2+}-mobilising effects of 2-MeSATP increased in VSMC during culture in serum indicating upregulation of $P2Y_1$ receptors in the transition from contractile to synthetic phenotype [35]. This was confirmed at the mRNA level were also the $P2Y_2$ receptor was upregulated while the $P2X_1$ receptor was totally downregulated [36]. Thus, mitogenic P2Y receptors are upregulated while ion-channel coupled receptors with only contractile effects are downregulated. Growth factors, cytokines and interestingly also ATP are potent stimulators of $P2Y_2$ receptor expression [37, 38]. Thus, factors of importance in the development of vascular disease increase mitogenic $P2Y_2$ receptors which is further supported by their upregulation in neointima after balloon angioplasty [39]. *In vivo*, in congestive heart failure $P2X_1$ receptors are downregulated in VSMC in resistance arteries but unchanged in the aorta indicating specific regulation depending on vascular region [40]. In endothelial cells shear stress downregulates $P2X_4$ receptor mRNA [41].

Haemostasis

The clinically so far most important contribution of drug development aimed at P2 receptors are the beneficial effects of the platelet ADP-receptor antagonist clopidogrel in atherosclerotic disease as has been demonstrated in the CAPRIE and CURE trials (CAPRIE) [42]. The effect is mediated by antagonism at the $P2Y_{12}$ receptor on platelets [43]. The platelets also express $P2Y_1$ receptors that have been shown in knockout mice to be of similar importance as $P2Y_{12}$ receptors [44]. This prothrombotic effect is counteracted by the effects of UTP and ATP on the endothelium that stimulates a substantial release of tissue-type plasminogen activator (tPA) with fibrinolytic effects [14]. To make the the picture even more complex, ATP also release plasminogen activator inhibitor (PAI-1) probably from platelets which in turn inhibits tPA [14]. Thus, P2 receptors are involved at several stages of haemostasis, which is important for the development of atherosclerosis and thrombotic occlusions leading to myocardial infarction and stroke.

Ectonucleotidases

A highly interesting area is the degradation pathways of extracellular nucleotides. Vascular ATP diphosphohydrolase (CD39) is an endothelial cell membrane protein with both ecto-ATPase and ecto-ADPase activities acting as a local apyrase [45]. Endothelial ADPase activity is lost following ischemia-reperfusion injury, xenograft rejection and inflammation that leads to increased levels of ATP and ADP [46]. This may cause platelet aggregation but also other effects such as mitogenic effects on VSMC. Infusion of systemic apyrase inhibits platelet aggregation and prolongs xenograft survival [47].

Conclusion

The extracellular nucleotides ATP, ADP, UTP and UDP are important for vascular regulation. They stimulate vasoconstriction, vasodilatation by release of prostaglandins, NO and EDHF, are involved in shear stress, are growth factors for VSMC's, stimulate platelet aggregation, tPA and PAI release. The effects are mediated by P2 receptors with at least 13 subtypes. The physiological effect is dependent on the release of extracellular nucleotides, the degradation by ectonucleotides, the type of P2 receptors expressed on the cells, their desensitization rates and their second messengers. P2 receptors have highly specific organ distribution and they can be rapidly up or down regulated. These mechanisms constitute an "extracellular nucleotide system" which may be of similar importance as the sympathetic and renin-angiotensin-aldosterone systems in cardiovascular regulation and pathophysiology.

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Effects of Large Registries (USRDS, DOPPS) on the Quality of Hemodialysis Therapy in the United States

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Since its beginning the United States Renal Data System had the goal to be more than a registry that counts the number of patients by treatment modality and their outcomes. In addition to providing such counts, it has put a major effort into characterizing details of comorbidities and treatment and seeking correlations with outcomes measures such as survival, hospital admissions and quality of life [1]. An ongoing large population-based study on random samples of dialysis units, the Dialysis Outcomes and Practice Pattern Study (DOPPS) has expanded on this clinically relevant research by focusing more directly on modifiable treatment practices [2]. This study has the goal of identifying practice patterns that are associated with better outcomes for hemodialysis patients, while adjusting for patient characteristics including comorbid conditions. Since its expansion to Japan and five large European countries (France, Germany, Italy, Spain and the United Kingdom), the DOPPS is representative of almost 80 percent of the hemodialysis population worldwide. The USRDS through several detailed "Special Studies" of random samples of hemodialysis patients and the DOPPS through its random sample of hemodialysis units have yielded clinically relevant outcomes information associated with treatment factors.

Specific examples of practice patterns from both studies that show a clear association with superior patient survival include the following: higher dialysis dose as measured by urea reduction ratio (URR) or Kt/V) [3,4], higher middle molecule clearance according to vitamin B12 at the same level of Kt/V for urea [5], high flux membranes, lower serum phosphorus level [6], higher body mass index [7,8], higher serum albumin [7] and better control of anemia [9]. Similar benefits on hospitalization can also be shown. Many more examples are evolving, particularly through the international component of the DOPPS [2]. For these observational studies a careful and detail statistical adjustment is necessary to minimize the role of confounding [10].

The practice in the US has markedly changed during the last decade. This has been documented by assessments in serial random samples of patients. The dialysis dose has increased by 50 percent yielding an average Kt/V of 1.48 in the year 2000. The use of high flux synthetic membranes has increased from 2% in 1990 to 27% in 1997 with a corresponding reduction of cellulose membranes [1]. It is hoped that the fraction of patients with a serum phosphorus of $>$6.5 mg/dL will be reduced from 40 percent in 1994, but documentation of such a response is still lacking. The control of anemia has improved to a large degree even several years after the effect of the first introduction of erythropoietin [1]. Although a cause and effect relationship cannot be proven, improved practices clearly followed documentation of significant correlation between clinically modifiable factors and mortality risk. Evidence based DOQI Guidelines have likely enhanced the impact of these reported results [11]. It is noteworthy that mortality rates for US hemodialysis patients have significantly improved during the same time period. As we await the results of major randomized clinical trials, such as the HEMO trial [12], observational studies are very useful, as they provide a wealth of information at a substantially lower cost than that of a trial. One may therefore conclude that epidemiological studies are useful for improving patient care and outcomes, particularly when they are population-based as is the case in USRDS, US-DOPPS. It is likely that these results from the US can be extrapolated to the Worldwide DOPPS.

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Renal and Endocrine Mechanisms in Obesity Hypertension

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Abstract

Obesity is associated with profound alterations of the cardiovascular system characterised by sodium and fluid retention and often an increase in systemic blood pressure. Abnormalities in renal sodium homeostasis have been related both to changes in renal structure and function. Functional changes may be related to obesity-induced stimulation of the sympathetic and renin-angiotensin systems. Increased activity of these systems may be

driven by factors directly derived from adipose tissue. Thus, leptin released from adipose tissue has been shown to stimulate sympathetic activity. Enhanced leptin-driven renal sympathetic outflow, in combination with low atrial natriuretic peptide plasma levels possibly due to over-expression of the natriuretic peptide clearance receptor in adipocytes, may enhance sodium retention and volume expansion. Furthermore, adipose tissue is now known as a rich site of angiotensinogen production and may also produce other substances of potential importance for cardiovascular function. This review discusses some of the recent findings on renal function and adipose-tissue derived endocrine factors in obesity hypertension.

Key Words

Fat cells, obesity, hypertension, renin-angiotensin system, sympathetic nervous system, leptin, kidney function

Introduction

Abdominal obesity is an important and independent risk factor for cardiovascular morbidity and mortality [1]. As a rule, excess fat mass is accompanied by increases in blood volume, heart rate, and cardiac output, alterations which are subsequently reflected in an increase in cardiac mass with or without systemic hypertension [2–5]. Apart from the frequently discussed role of insulin resistance and increased sympathetic activity [6], several more recent pathophysiological views on obesity-associated hypertension have emerged. These include the strong emphasis on mechanisms related to changes in sodium balance [7] and the more recent hypothesis that adipocyte-derived factors may directly contribute to these alterations [8]. In this paper, we briefly review the obesity-related alterations in renal and endocrine function and their possible contributions to the pathophysiology of obesity hypertension.

Renal Mechanisms in Obesity-associated Hypertension

At early stages, a gain of body weight is usually associated with an increase in plasma volume and a concomitant decrease in peripheral resistance [9–11]. At later stages, obesity-associated hypertension is not different from "essential" hypertension in that total peripheral resistance is increased, a finding that may be explained by structural changes in the vessel walls and enhanced vascular reactivity. These changes may be secondary to long-standing volume overload and endothelial dysfunction, resulting in a misbalance between vasodilatory and vasoconstrictive substances in the blood vessel wall [12,13].

When challenged with saline infusions, obese subjects have a lesser natriuretic response than lean individuals [2,14,15], suggesting a rightward shift of the pressure natriuresis curve. This finding implies that obese individuals may require a higher mean arterial pressure in order to excrete a comparable amount of sodium. The rightward shift of the pressure-natriuresis curve may result both from structural and functional changes within the kidney. Structural changes in the kidney are associated with increased intrarenal fat, increased extracellular matrix accumulation and with increased intrarenal pressure [12,16–18]. The adipocyte-derived cytokine/hormone leptin was recently shown to induce proliferation of glomerular endothelial cells and increase the deposition of collagen type IV, increase the expression of transforming growth factor-β, and promote proteinuria [19]. Thus, leptin appears to have profibrotic properties that may in part explain the changes in renal histology associated with obesity [20,21]. Functional alterations include increased activity of the renin-angiotensin-aldosterone system (RAAS) and increased activity of the sympathetic nervous system [22,23].

Several investigators have reported increased sympathetic activity in obese hypertensive individuals [6,24]. Masuo *et al*. [25] also reported a greater increase in heart rate and plasma norepinephrine levels in individuals who experienced a rise in blood pressure following weight gain compared to individuals with similar weight gain but no concomittant increase in blood pressure. Likewise, in a recent study, we noted higher catecholamine levels in hypertensive obese compared to age and sex matched normotensive obese individuals [26]. In humans, renal norepinephrine spillover, a marker of renal sympathetic nerve activity, is significantly correlated with BMI [27,28]. The role of renal innervation in obesity-related sodium reabsorption is also illustrated by bilateral renal-denervation in animal models of obesity-associated hypertension. Neither the high-fat diet dog model [29], nor the chronic hyperinsulinemia rat model [30], develop hypertension or sodium retention when sympathetic innervation of the kidney is destroyed. Together, these findings clearly suggest a role of increased renal sympathetic nervous activity in obesity-associated sodium retention and volume expansion.

Leptin

Circulating leptin levels correlate with body fat mass [31–33] and higher plasma leptin levels were associated with fat cell hypertrophy [34]. Several investigators have now also reported higher circulating levels of leptin in hypertensive subjects, even when statistically corrected for BMI [35–39]. Increased leptin levels in human subjects also predict increased heart rate [38], hyperinsulinemia [36–38], or increased myocardial wall thickness [39]. Medical history of myocardial infarction [40] or a family history of essential hypertension [41] were likewise positively associated with increased circulating leptin levels, as were parameters of the renin-angiotensin-aldosterone system [35,42,43].

In Wistar rats, intracerebroventricular administration of leptin increases mean arterial pressure and vascular resistance [44]. Furthermore, SNS activity to the kidney and other organs was increased, a finding that may explain increased peripheral vascular resistance. Interestingly, renal vascular resistance was not changed despite increased renal SNS activity [44]. In Sprague Dawley rats, administration of leptin for 7 days increased mean arterial pressure by 7 mm Hg and heart rate by 20-30 beats/min [45], irrespective of the site of infusion (carotid artery vs. femoral vein). These cardiovascular actions of leptin are further enhanced by the inhibition of nitric oxide formation [46]. Acute infusions (3 h) of much higher leptin doses increased SNS outflow to brown adipose tissue, to the kidney, to the hindlimb, and to the adrenals in the Sprague Dawley, but not in the obese Zucker rat, which harbors a defective leptin receptor [47]. These acute effects of leptin administration on the renal sympathetic nervous system are primarily due to the activation of the hypothalamic melanocortin system, whereas the possible thermogenic effects on the sympathetic system of brown adipose tissue are not [48]. Leptin-deficient obese mice, despite an enormous accumulation of body fat, have lower blood pressure compared to lean littermates [49]. In contrast, the hyperleptinemic agouti yellow mouse, carrying a genetic defect that disrupts signalling from the melanocortin-4 receptor, has higher blood pressure

compared to lean littermates, despite a low degree of obesity [49]. In transgenic "skinny" mice over-expressing leptin in the liver, blood pressure was markedly higher than in nontransgenic littermates [50] and was lowered by sympatholytic agents, suggesting a prime role of the SNS in leptin-associated blood pressure elevation.

Interestingly, affected members of a Turkish family with a monogenic form of leptin deficiency have normal resting blood pressure, but display postural hypotension and an attenuated blood-pressure response to the cold-pressure test, suggesting impairment of the SNS response to these stimuli [51]. These findings are in agreement with a recent human study that found no differences in heart rate or blood pressure, but an exaggerated SNS response to orthostasis and an increase in heart rate variability, suggestive of higher SNS activity, in subjects from the highest vs. those from the lowest plasma leptin quartile [52].

In contrast to the centrally mediated sympatho-excitory effects leptin may have direct vasodilatory effects. Thus in anaesthetised Wistar rats, in the presence of ganglion blockade, single bolus injections of leptin resulted in a hypotensive response possibly attributable to the formation of nitric oxide [53]. In the kidney, short-term leptin infusion into the renal artery of normotensive rats resulted in ipsilateral, but not contralateral increases of sodium excretion and urine volume [54,55]. In contrast, long-term leptin infusion had no effect on urine volume and sodium excretion, but increased renal plasma flow, renal vascular resistance, and systemic blood pressure [45]. The failure of the kidneys to increase sodium excretion in the high blood pressure situation suggests a rightward shift of pressure natriuresis due to long-term leptin infusion. It is interesting to note that similar shifts in pressure natriuresis are a common pathophysiological finding in obesity-associated hypertension and have been linked to increased tubular sodium reabsorbtion (see discusion above and [7]). In spontaneously hypertensive rats, leptin failed to induce diuresis, but the response was restored in rats by renal denervation, suggesting that increased sympathetic activity may have abolished the diuretic effect of acute leptin infusion [56].

The Renin-Angiotensin-Aldosterone System

Although obesity represents a condition of volume expansion and sodium retention, the RAAS appears to be inadequately activated in obesity (recently reviewed in [57]). Our group reported a significant relationship between plasma leptin and plasma AGT levels in humans [43] and a relationship with plasma leptin was recently also described for PRA [42]. In our study, about 20% of plasma AGT variance was explained by leptin, thereby suggesting that adipose tissue may directly contribute to circulating AGT levels.

Adipocytes are now known to form all components of the RAS, including AGT and Ang II [57]. In hypotensive *AGT*-knock out mice with undetectable plasma AGT levels, adipose tissue specific expression of a transgenic construct (*aP2*-promoter (adipocyte specific fatty acid binding protein) + *AGT* gene) resulted in detectable plasma AGT levels (about 10% of wildtype) and normalisation of blood pressure [58]. In another study, *in vivo* adipose tissue *AGT* expression in rats was significantly reduced by fasting and markedly increased by refeeding [59]. These changes in *AGT* expression in adipose tissue were accompanied by parallel changes in blood pressure, which fell on fasting and increased during refeeding [59]. In human subjects a positive correlation between visceral or subcutaneous adipose tissue *AGT* expression and BMI [60,61] or waist-to-hip ratio (reflecting abdominal obesity) [62] have been reported. AGT secretion from isolated human adipocytes has also been shown to correlate with adipocyte volume or BMI of the donors [63].

Based on the current data, we hypothesize that large adipose tissue depots contribute to plasma AGT levels and that perivascular adipose tissue may locally modulate vascular reactivity. The contribution of Ang II to adipocyte metabolism related to obesity-associated hypertension and to adipose tissue blood flow regulation is unclear at present, as only preliminary evidence is available suggesting that adipose tissue blood flow and lipolysis, both measured by the microdialysis technique, are decreased by Ang II [64]. Thus, further studies will need to clarify the pathogenic role of the adipose tissue RAS in obesity-associated hypertension.

Natriuretic Peptide System

The natriuretic peptide system consists of three peptide hormones (atrial natriuretic peptide – ANP, brain natriuretic peptide – BNP, C-type natriuretic peptide – CNP) and three natriuretic peptide receptors, including the homodimeric clearance receptor, NPr-C, that does not stimulate cGMP formation and probably has a role in regulation of ANP availability. A potential role of this system in the development of obesity-associated hypertension has recently been proposed by Dessì-Fulgheri and co-workers: In rats, fasting selectively inhibits adipose tissue *NPr-C* but not *NPr-A* gene expression [65] and *NPr-C* gene expression is enhanced in human adipose tissue of obese hypertensive but not obese normotensive subjects [66]. These findings suggest that increased expression of the *NPr-C* receptor gene in adipose tissue may directly influence the systemic availability

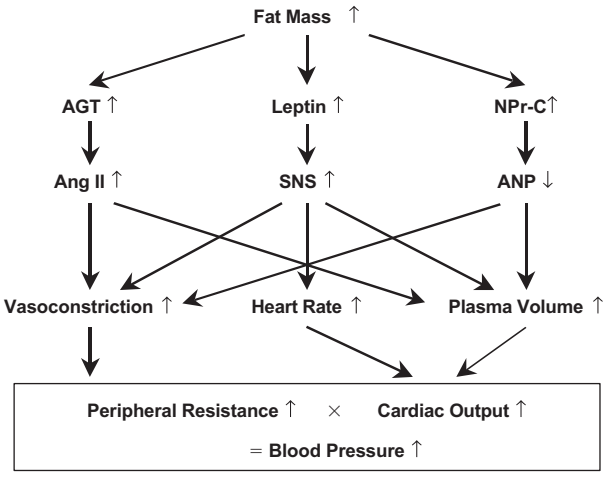

Fig. Putative contribution of adipose tissue to obesity-associated hypertension. Both, angiotensinogen (AGT) and angiotensin II (Ang II) are formed by adipose tissue and may contribute to higher plasma AGT levels as well as enhanced vascular tone. Leptin secretion from adipocytes enhances sympathetic nervous system activity, which may contribute to enhanced vascular tone, but also to increased cardiac output (by increasing heart rate and blood volume). Expression of the natriuretic petide clearance receptor (NPr-C) in adipose tissue may contribute to low plasma levels of the atrial natriuretic peptide (ANP), which in turn promotes sodium retention and volume expansion. Vasoconstriction increases peripheral resistance, which together with increased cardiac output leads to high blood pressure in susceptible individuals.

and thus action of natriuretic peptides in obese individuals. This hypothesis would be compatible with the recent observation of lower ANP plasma levels in obese hypertensive compared to obese normotensive subjects (along with elevated PRA and plasma aldosterone in the hypertensive group) [66]. In addition, these obese hypertensives demonstrated a stronger responses to exogenous ANP, as measured by blood pressure reduction and natriuresis, at the end of a low-calorie diet compared to baseline [67]. Furthermore, the ANP response to a saline load as well as the suppression of PRA and plasma aldosterone were significantly blunted in obese subjects [15]. Thus, increased expression of NPr-C in adipose tissue may counteract the natriuretic and vasodilatory effects of natriuretic peptides and may thus promote the development of obesity-associated hypertension

Conclusion

The current view of the pathophysiology of obesity-associated hypertension includes sodium retention and volume expansion, increased SNS activity, hyperleptinemia, and activation of the RAAS. It is important to note that adipose tissue itself may play a crucial role in the pathophysiology of obesity-associated (summarized in the figure). The importance of adipocytes for obesity-associated hemodynamic changes may, however, not only be related large adipose tissue depots, but also to the adipocytes lining the vascular walls or surrounding organs like the heart or kidney. Further studies will be needed to examine the relationship between adipose tissue and its contribution to renal and endocrine dysfunction in obesity hypertension.

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Molecular Genetics of Renal Tubular Disease

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Key Words

Renal tubular transport, genetic renal disease

Monogenic Tubular Diseases: Gene Identification

Exciting new insights into the physiology and pathophysiology of renal tubular transport have resulted from recent identification of genes responsible for inherited renal tubular diseases. Virtually all renal tubular disorders are monogenic diseases, in which, in a given patient the disease is caused by a defect in a *single* gene only. Thus a single mutation in one or both alleles (dominant or recessive) of one gene alone is sufficient to cause the disease. For some of these disorders genetic locus heterogeneity is known, which is to say, that in *different* patients a similar or identical disease phenotype can be caused by *different* genes. Although gene identification in monogenic disorders by

Table 1: Major symptoms of renal tubular disorders, resulting from defects in disease genes expressed in specific tubular segments

Proximal tubule	
renal Fanconi syndrome:	
altered reabsorption:	Hyperaminoaciduria, glucosuria, hypophosphatemia, hypouricemia, acidosis
clinical symptoms:	Polyuria, dehydration, rickets, growth retardation, tubular proteinuria
Medullary thick ascending limb of Henle's loop (mTAL)	
severe salt loss, polyuria, hypokalemic metabalic alkalosis, hypercalciuria	
Distal convoluted tubule	
salt loss, hypokalemic metabolic alkalosis, hypomagnesemia, hypocalciuria	
Collecting duct	
polyuria, hypo-/hypernatremia, hypo-/hyperkalemia, acidosis	

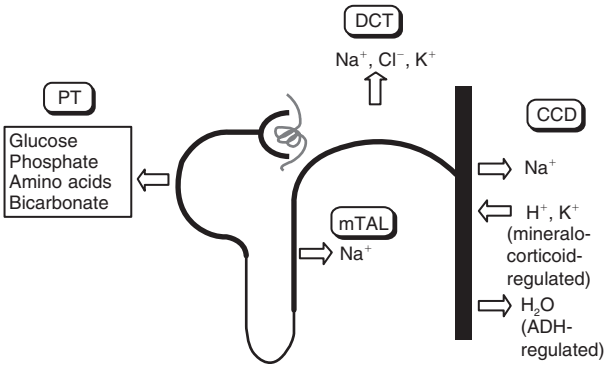

Fig. 1. Segment-specific tasks of tubular reabsorption is conveyed by segment-specific gene expression of the respective transport proteins (PT = proximal tubule; mTAL = medullary thick ascending limb of Henle's loop; DCT = distal convoluted tubule; CCD = cortical collecting duct)

positional cloning is a very laborious process, it offers the unique opportunity of directly clarifying the primary cause (i.e. the etiology) of the disease. Functional studies in patients with monogenic disorders of renal tubular ion transport will help to elucidate the pathophysiology of such disorders and to potentially open new inroads into therapy.

In this review primary renal tubular disorders are discussed, in which the responsible genetic defect has already been elucidated. We follow the route along the nephron, since each tubular segment serves characteristic purposes in tubular transport. If this function is impaired, due to mutations in a gene expressed in this segment, a group of symptoms characteristic for this tubular segment results (Table 1). In all tubular segments transtubular reabsorption is driven by the primary active (ATP consuming) transport of the basolateral Na^+/K^+ ATPase. The resulting luminal to intracellular Na^+ gradient constitutes the driving force for secondary active transport systems in the luminal plasma membrane. Secondary active luminal transport is coupled to transport of different solutes like electrolytes, sugars, amino acids etc.. On the luminal side, expression of genes for different transport systems are expressed in a segment specific fashion, thus conveying tubular segment specificity of transport (Figure 1).

Proximal Tubule

Major findings in primary tubulopathies of the proximal tubule are glucouria, phosphaturia, amino aciduria and proximal tubular acidosis. The combination of multiple of these proximal tubular reabsorption defects is summarized under the term "renal Fanconi syndrome" or "Debré-DeToni-Fanconi syndrome". Genetic disorders of proximal tubular reabsorption are summarized in Table 2.

Renal glucosuria: This transport disorder represents a very selective defect of renal glucose reabsoprtion in the proximal tubule. *Disorders of renal phosphate reabsorption* are expressed in the proximal tubule. The etiology of several disorders of renal phosphate reabsorption has been elucidated as listed in Table 2. *Cystinuria* represents an autosomal recessive group of diseases, in which reabsorption of the dibasic amino acids cystin, ornithin, arginin and lysin is disturbed, leading to recurrent urinary calculi. About 44% of all cases are due to mutations in the *SLC3A1* gene (cystinuria type 1). *Proximal renal tubular acidosis*: In the proximal tubule, H^+ secretion is coupled to Na^+ reabsortion through the action of the luminal Na^+/H^+ exchanger (NHE3). Carbonic anhydrase 2 (CA 2) is involved in Na^+ reabsorption across this route by at least one luminal and two intracellular catalytic processes. Thus, carbonic anhydrase inhibition by the mild diuretic acetazolamide, and likewise a genetic defect in CA 2, which occurs in *osteopetrosis with renal tubular acidosis*, result in net fluid loss and Na^+ loss with the clinical picture of proximal tubular acidosis (Table 2). *Dent's disease* follows X-chromosomal inheritance and leads in male patients to renal Fanconi syndrome with nephrocalcinosis and nephrolithiasis. End-stage renal disease may ensue in early adulthood.

Medullary Thick Ascending Limb of Henle's Loop (mTAL): Bartter Syndrome

Reabsorption defects of the mTAL present clinically as Bartter syndrome (BS) [Bartter *et al*., 1962; Barakat *et al*., 1974; Fanconi *et al*., 1971; Hogewind *et al*., 1981; Schwarz and Alon, 1996; Stein, 1985]. Characteristic findings are polyuria, renal salt loss, and hyperreninemic hyperaldosteronism with metabolic alkalosis. Paradoxically, despite hyperaldosteronism, there is low or normal blood pressure due to the concomitant salt loss. Hypomagnesemia and hypercalciuria with or without the complication of nephrocalcinosis and may be present.

Within Bartter syndrome at least three distinct clinical phenotypes can be distinguished (Table 3): (i) The severe antenatal hypercalciuric variant, antenatal Bartter syndrome (aBS) is a life threatening disease, which through intrauterine polyuria leads to polyhydramnios with prematurity and to severe neonatal polyuria, salt loss, dehydration with fever, hypercalciuria with nephrocalcinosis and hyperprostaglandiuria [Ohlsson *et al*., 1984; Seyberth *et al*., 1985; Proesmans *et al*., 1985]. (ii) The "classic" subtype (cBS) with onset beyond the neonatal period is following a much milder course, with later clinical onset and the absence of nephrocalcinosis. (iii) A third variant of aBS follows a severe course and in addition may result in end-stage renal disease in early childhood. This form is invariably associated with sensorineural deafness and has therefore been termed "Bartter syndrome with sensorineural deafness (BSND)" [Brennan *et al*., 1998; Jeck *et al*. 2001].

For Bartter syndrome mutations in four genes have been identified as causative for the antenatal subtype [Rodriguez-Soriano, 1998]. The resulting classification is summarized in Table 4. The products of all four genes are involved in chloride reabsorption in the medullary thick ascending limb (mTAL) of Henle's loop [Hebert and Andreoli, 1984]. In *Bartter syndrome type 1* the function of the $Na^+/K/^+2Cl^-$ cotransporter (*NKCC2*) of the

Table 2: Primary tubulopathies of the *proximal tubule (PT)*

Disease (Inheritance)	Gene responsible (Chromosome)	Gene product	Clinical presentation
Glucose reabsorption			
Renal glucosuria (AR)	*SLC5A2* (16p11.2) *SLC5A1* (22q13.1)	SGLT2 SGLT1	renal glucosuria type A, renal glucosuria type B, glucose-galactose malabsorption
Phosphate reabsorption			
Pseudohypoparathyroidism type 1A (Albright syndrome)	*GNAS1* (20q13.2)	α-subunit of a G_s-protein	bone changes, hypergonadotropic hypogonadism
Pseudohypoparathyroidism type 1B	*GNAS1* (20q13.2) (imprinted)	parathormone receptor?	osteopenia, hypercalciuria
Amino acid reabsorption			
Cystinuria type 1 (AR)	*SLC3A1* (2p16.3)	dibasic amino acid transporter	urinary calculi (cystin calculi)
Cystinuria type 2 (AR)	*SLC7A9* (19q13.1)	dibasic amino acid transporter subunit b(0, +)AT	urinary calculi (cystin calculi)
Lysinuric protein intolerance	*SLC7A7* (14q11.2)	amino acid transporter	mental & physical retardation, urinary excretion of lysine, mild intestinal malabsorption
Proximal renal tubular acidosis (pRTA)			
Osteopetrosis with renal tubular acidosis (AR)	(8q22)	carbonic anhydrase 2	osteosclerosis at 2 years proximal RTA, nephrolithiasis, osteopetrosis, growth retardation
Other proximal tubular reabsorption defects			
Dent's disease (X-linked)	*CLCN5* (Xp11.22)	chloride channel	renal Fanconi syndrome with nephrolithiasis, nephrocalcinosis, renal failure, hypercalciuria, phosphaturia, microglobulinuria

AR = autosomal recessive; AD = autosomal dominant; XR = X-chromosomal recessive; (updated information on clinical and molecular genetics of these diseases is found at *http://www3.ncbi.nlm.nih.gov/Omim/searchomim.html*)

Table 3: Clinical symptoms and laboratory findings in Bartter- and Gitelman syndromes[1]

Clinical variant	Onset	Symptoms	Laboratory findings	Complications
Bartter syndrome (BS)				
antenatal BS	antenatal	Dehydration, fever, prematurity, growth retardation	Hypercalciuria, ± hypomagnesemia	Polyhydramnios, neprocalcinosis
"classic" BS	childhood	Mild course	Usually no hypercalciuria	No nephrocalcinosis
antenatal BS with sensorineural deafness	antenatal	Dehydration, fever prematurity, growth retardation, sensorineural deafness	Hypercalciuria, ± hypomagnesemia	Polyhydramnios, nephrocalcinosis sensorineural deafness
Gitelman syndrome	Adolescence	Fatigue, tetany	Hypomagnesemia, hypocalciuria	Chondrocalcinosis

[1]Common to all disease variants are the characteristic symptoms of: hypokalemic meatabolic alkalosis, secondary hyperreninemic hyperaldosteronism in the presence of normal or low blood pressure

medullary thick ascending limb of Henle's loop (mTAL) is abolished [Simon *et al*., 1996b; Vargas-Poussou *et al*., 1998; Kurtz *et al*., 1997]. Bartter syndrome type 1 therefore represents a naturally occurring parallel of furosemide action [Köckerling *et al*., 1996]. In *Bartter syndrome type 2* mutations in the inwardly rectifying luminal ATP-sensitive potassium channel Kir 1.1 (ROMK) [Ho *et al*., 1995; Shuck *et al*., 1994] have been shown to cause an identical disease phenotype as described for *NKCC2* [Simon *et al*., 1996c; International Collaborative Study Group for Bartter-Like Diseases, 1997; Vollmer *et al*., 1997; Vollmer *et al*., 1998]. The contribution of ROMK to the disease can be explained by its secretory function in the mTAL, where it is recycling potassium across the apical membrane, thus ensuring efficient function of the $Na^+/K^+/2Cl^-$ cotransporter [Greger and Schlatter, 1981].

In *Bartter syndrome type 3* mutations and deletions in the recently discovered gene for the chloride channel *CLCNKB* [Kieferle *et al*., 1994; Adachi *et al*., 1994; Saito-Ohara *et al*.,

Table 4: Primary tubulopathies of the medullary thick ascending limb of Henle's loop (mTAL) and the distal convoluted tubule (DCT)[1]

Disease (Inheritance)	Gene responsible (Chromosome)	Gene product	Clinical presentation (diuretic effect mimicked)
Medullary thick ascending limb of Henle's loop (mTAL)			
Bartter syndrome (BS)			
BS type 1	*SLC12A1* (15q15–q21)	Furosemide-sensitive $Na^+/K/^+2Cl^-$ cotransporter (NKCC2)	Antenatal BS (furosemide)
BS type 2	*KCNJ1* (11q24–q25)	luminal ATP-regulated K^+-Kanal (ROMK)	Antenatal BS
BS type 3	*CLCNKB* (1p36)	basolateral Cl^- channel (CLC-Kb)	Antenatal and "classic" BS
BSND (BS with sensorineural deafness)	(1q32.3)	regulator of a channel?	antenatal BS with sensorineural deafness
Familial hypomagnesemia with hypercalciuria and nephrocalcinosis (AR)	*PCLN1* (3q27)	Paracellin-1	hypomagnesemia with hypercalciuria and nephrocalcinosis
(AD)	*FXYD2* (11q23)	γ-subunit of Na^+/K^+ ATPase	hypomagnesemia
Distal convoluted tubule (DCT)			
Gitelman syndrome	*NCCT* (16q13)	Thiazide-sensitive Na^+/Cl^- cotransporter	Gitelman-syndrome (thiazides)

[1]All diseases in this table follow autosomal recessive inheritance (Updated information on clinical and molecular genetics of these diseases is found at *http://www3.ncbi.nlm.nih.gov/Omim/searchomim.html*)

Table 5: Primary tubulopathies of the *collecting duct (CD)*

Disease (Inheritance)	Gene responsible (Chromosome)	Gene product	Clinical presentation
Disturbances of water reabsorption			
Nephrogenic diabetes insipidus (XR)	*AVPV2R* (Xq28)	Vasopressin-V2-receptor	Polyuria and polydipsia
(AR, rarely AD)	*AQP2* (12q12–q13)	Aquaporin-2-water channel	Polyuria and polydipsia
Disturbances of sodium and potassium reabsorption			
Pseudohypoaldosteronism type 1, renal type (AD)	*MLR* (4q31.1)	Mineralocorticoid receptor	Propensity to hyperkalemia, acidosis salt loss
Pseudohypoaldosteronism type 1 multiple type (AR)	*SCNN1A, B, G* (12p, 16p, 16p)	α-, β-, γ-subunit of the epithelial Na^+ channel (ENaC: loss of function)	life threatening salt loss in the newborn
Liddle syndrome	*SCNN1B, G* (16p13, 16p12)	α- or -γ-subunit of the epithelial Na^+ channel (ENaC: gain of function)	hypertension, hypo, kalemic $\downarrow$ alkalosis Plasma: $\downarrow$ renin, aldosterone, $\downarrow$ angiotensin
Pseudohypoaldosteronism type 2 (Gordon syndrome) (AD)	*PHA2A* (1q31–q42) *PHA2B* (17p11–q21) *PHA2C* (12p)	? ?	Hypertension, hyperkalemia, hyperchloremic acidosis
Distal renal tubular acidosis (dRTA)			
dRTA (AD)	*AE1 (SCL4A1)*	Na^+/bicarbonate exchanger	dRTA
dRTA (AR) with sensorineural deafness	*ATP6B1* (2p13)	B1-subunit of the vacuolar H^+-ATPase	dRTA with sensorineural deafness
dRTA (AR) with preserved hearing	*ATP6N1B*	subunit of the vacuolar H^+-ATPase	dRTA with preserved hearing

AR = autosomal recessive; AD = autosomal dominant ;XR = X-chromosomal recessive; (Updated information on clinical and molecular genetics of these diseases is found at http://*www3.ncbi.nlm.nih.gov/Omim/searchomim.html*)

1996; Takeuchi *et al*., 1995; Uchida *et al*., 1992; Uchida *et al*., 1995; Vandewalle *et al*., 1997] are responsible for the phenotype [Simon *et al*., 1997; Konrad *et al*., 2000]. While Bartter syndrome types 1, 2 and BSND as a rule present as the severe, antenatal form, BS type 3 in many instances presents as the milder "classic" form with the absence of hypercalciuria or nephrocalcinosis [Simon *et al*., 1997; Bettinelli *et al*., 1992]. Gene identification in Bartter syndrome has lead to important insights into renal salt handling, diuretic action, and blood pressure regulation [Lifton 1996] (Figure 3).

Very recently we have identified by positional cloning the gene responsible for *Bartter syndrome with sensorineural deafness (BSND)* (Birkenhäger *et al*., submitted). The gene is novel, i.e. it is completely unrelated to any known genes. In the kidney it is expressed in mTAL as well as inner medulla and also in ndolymph-producing cells of the inner ear. Since the gene product, barttin, contains two putative transmembrane domains, this novel protein might be a regulator of one of the transporters involved in BS, or else, might represent a novel transporter itself. Identification of genes responsible for inherited hypomagnesemia

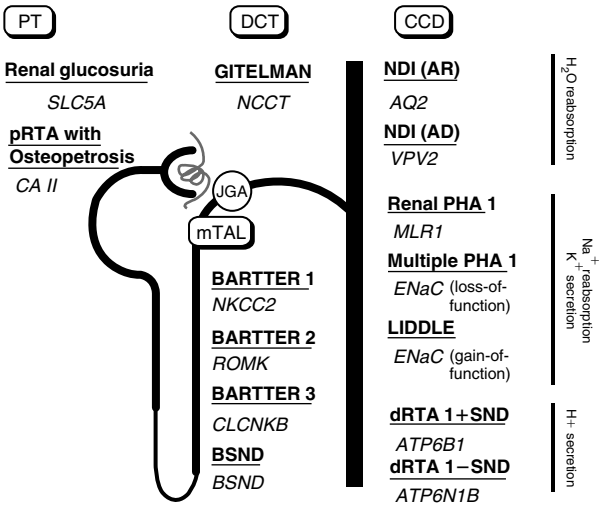

Fig. 2. Expression of genetic defects in relation to distinct tubular segments. (PT = proximal tubule; mTAL = medullary thick ascending limb of Henle's loop; DCT = distal convoluted tubule; CCD = cortical collecting duct; SND = sensorineural deafness; JGA = juxtaglomerular apparatus; BSND = Bartter syndrome with sensorineural deafness (SND); AR = autosomal recessive; AD = autosomal dominant.) Diseases of renal tubular transport are underlined, names of responsible gene are shown in italics (see text).

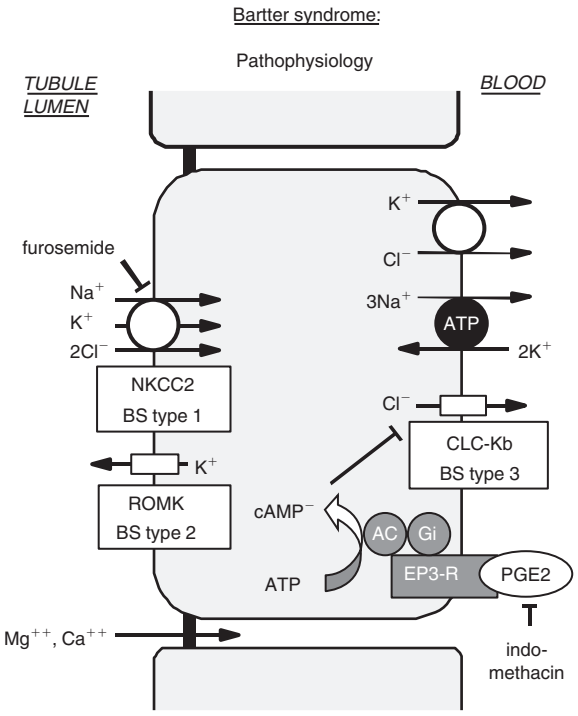

Fig. 3. Expression in a cell of the medullary thick ascending limb of Henle's loop (mTAL) of ion transport proteins that are defective in Bartter syndrome. Na^+/Cl^- reabsorption in the mTAL is driven by the primary active Na^+/K^+ ATPase. Na^+ and Cl^- follow on the luminal side via the secondary active furosemide-inhibitable $Na^+/K^+/Cl^{2-}$ cotransporter (NKCC2), which is defective in BS type 1. The driving force for this cotransporter is strongly dependent on K^+ recycling back to the lumen through the ATP-regulated, inwardly-rectifying K^+ channel ROMK [Greger and Schlatter, 1981]. Genetic defects in ROMK are responsible for BS type 2. Both defects lead to the severe antenatal form of Bartter syndrome aBS. In this way, BS types 1 and 2 are the genetic equivalent of furosemide action. Cl^- exits from the cell on the basolateral side via a basolateral Cl^- channel CLCNKB and via a K^+/Cl^- cotransporter. Mutations in the *CLCNKB* gene are responsible for BS type 3. Defects in *CLCNKB* can lead to both, severe aBS as well as mild "classic" BS. In all forms of BS severe salt loss initiates a vicious circle, since extracellular volume contraction and reduction of glomerular filtration rate leads to increased PGE2 production, which through its EP3 receptor (an inhibitory G protein-coupled receptor) leads to a decrease of intracellular cAMP, which inhibits of the basolateral chloride channel. Salt and fluid supplementation with or without administration of cyclooxgygenase inhibitors (like indomethacin) are the adequate therapeutic strategies to break this vicious circle. Finally, diminished Cl^- reabsorption leads to lowering of the transtubular electrochemical gradient and hence to reduced paracellular Ca^{2+} and Mg^{2+} reabsorption. This might explain the hypercalciuria and occasional hypomagnesemia of aBS.

have recently been identified. This has lead to new insights into tubular magnesium handling [Simon *et al*., 1999; Meij *et al*., 1999; Meij *et al*., 2000]

Distal Convoluted Tubule: Gitelman Syndrome

The hypocalciuric-hypomagnesemic variant described by Gitelman *et al*. is distinct from Bartter syndrome by the predo inance of hypomagnesemia, muscular weakness and tetany [Gitelman *et al*., 1996] and represents a defect of the DCT (Table 1). For *Gitelman syndrome* only one gene is responsible: The gene for the thiazide-sensitve Na^+/Cl^- cotransporter (NCCT), which is expressed in the distal convoluted tubule (Figure 2, Table 3) [Simon *et al*., 1996a]. Since the Na^+/Cl^- cotransporter represents a receptor for thiazides, symptoms of Gitelman syndrome are mimicking the effect of thiazide diuretics, and Gitelman syndrome, therefore, can be considered a naturally occurring counterpart of chronic thiazide action.

Collecting Duct

The major role of the collecting duct (CD) lies in regulation of water, sodium, potassium and acid reabsorption or excretion. Accordingly, genetic defects in transport proteins expressed in the CD lead to the characteristic clinical signs and laboratory findings of severe polyuria (nephrogenic diabetes insipidus), disturbances of sodium and/or potassium homeostasis and metabolic acidosis (distal renal tubular acidosis) (Table 1).

The responsible genes for many disorders of renal transport expressed in CCD have been identified (Table 5): In X-chromosomal dominant *nephrogenic diabetes insipidus* (NDI) principal cells of the CD do not respond to vasopressin by antidiuresis, due to mutations in the gene for the vasopressin V2-receptor (Table 5). A rare recessive form of NDI is caused by defects in the gene for a aquaporin 2 (*AQP2*). This water channel is inserted into coated pits of the luminal membrane of principal cells in response to ADH, thereby greatly enhancing water permeability and allowing water reabsorption along the lumen to blood osmotic gradient in the inner medulla.

Pseudohypoaldosteronism type 1 (PHA1) of the renal type is characterized by severe sodium loss and life-threatening hyperkalemia in the newborn. There is unresponsiveness to mineralocorticoids

and marked excess of aldosterone and renin [Donnell *et al*., 1959; Oberfield *et al*., 1979; Kuhnle *et al*., 1990; Rosler, 1984; Bosson *et al*., 1986; Hanukoglu *et al*., 1994; Chung *et al*., 1995]. Salt supplementation often can be discontinued after infancy, even though aldosterone excess is persistent. Loss-of-function mutations in all 3 subunits of the epithelial sodium channel (ENaC) have been revealed as causative (Fig. 2, Table 5) [Chang *et al*., 1996; Strautnieks *et al*., 1996a; Strautnieks *et al*., 1996b].
Pseudohypoaldosteronism type 1 of the multiple type follows autosomal dominant inheritance, takes a milder course than the renal type and remits with age [Geller *et al*., 2000].
Liddle syndrome represents the clinical and pathophysiologic mirror image of PHA1 multiple type, since it is caused by gain-of-function mutations in the alpha of gamma subunit of ENaC [Hansson *et al*., 1995; Chang *et al*., 1996]. The disease is characterized by hypertension with hypokalemic alkalosis, and decreased renin and angiotensin [Liddle *et al*., 1963] (Figure 2, Table 5). In Liddle syndrome, mutations delete a short proline-rich segment at the C-terminus of ENaC, which is necessary for endocytosis of channel molecules from the apical membrane. Therefore, in Liddle syndrome a high number of sodium channel subunits is locked into the apical membrane. This leads to enhanced renal sodium absorption and consecutively to hypertension [Snyder *et al*., 1995]. The genetic defect in autosomal dominant *pseudohypoaldosteronism type 2* (Gordon syndrome) has not yet been elucidated. Gordon syndrome is characterized by hypertension, hyperkalemia, metabolic acidosis and reduced plasma renin activity.
In distal renal tubular acidosis type 1 (dRTA1) proton secretion of "-intercalated-cells" in the cortical and medullary CD is defective. Urine pH can be diminished below 6.0, and there is loss of bicarbonate and decreased excretion of titratable acid and ammonium ions. Autosomal dominant dRTA1 is caused by defects in the gene (*AE1*) for the anion exchanger. Recessive forms with and without sensorineural deafness (SND) are caused by mutations in two different subunits of a vacuolar H^+-ATPase [Karet *et al*., 1999; Smith *et al*., 2000].

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Genetic Determinants of Renal Disease

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Genes in Search of a Function

Recent years have greatly advanced our knowledge of monogenetic renal diseases. In many instances, identification of the underlying genetic defect and positional cloning of the cognate gene has helped us to understand the pathogenesis of complex renal diseases. An excellent example are the mutations of tubular epithelial ion transporters that cause the complex changes and complications of Bartter's and Gittleman's syndromes. However, the pathogens of several well-defined monogenetic renal diseases are still poorly understood. Although their genes have been identified several years ago, the function of these genes remains unknown. One example are cystic renal diseases, a common cause for end-stage renal disease. This review will summarize some of the recent advances in cystic syndromes.

Autosomal Dominant Polycystic Kidney Disease

Autosomal dominant polycystic kidney disease is a common hereditary disease. Currently, mutations of two genes, PKD1 and PKD2, are known to cause the disease. These two genes encode for the distantly related proteins, polycystin-1 and polycystin-2. Polycystin-1 is a large integral membrane protein with 11 transmembrane domains, and a large extracellular domain [1,2]. Based on the presence of a C-type lectin domain, two leucine-rich repeat, and other structural elements, it has been speculated that polycystin-1 is a cell adhesion molecule that interacts with cell-surface or cell-matrix proteins. Most of the extracellular portion of polycystin-1 consists of Ig-like PKD repeats, a novel protein domain with a ß sandwich and a conserved WDFGDGS motif [3]. Since these repeats form homodimers, one possible ligand of polycystin-1 might be polycystin-1 itself [4]. Polycystin-2 in an integral membrane protein with six transmembrane domains, and similarity to voltage-gated ion channels (reviewed in [5]). It interacts with the coiled-coil domain of polycystin-1; this interaction appears to be a mandatory prerequisite for the translocation of polycystin-2 to the plasma membrane [6]. Polycystin-2 as well as a related protein, PKD2L, form calcium-permeable, non-selective cation channels [6–8]. Polycystin-2 also interacts with trp-channels [9] and hax-1 [10], a protein mediating a link to the actin cytoskeleton; however, the functional consequences of these interactions are currently unknown. Overexpression of polycystin-1 triggers tubulogenesis [11], a function that requires the carboxy-terminal 112 amino acids of polycystin-1 (manuscript submitted). Whether tubulogenesis mediated by polycystin-1 is related to its ability to activate protein kinase C and/or inhibit degradation of ß-catenin is currently under investigation [12,13]. Targeted disruption of either PKD1 or PKD2 causes embryonal lethality in homozygote animals [14,15]. While both animal models develop renal cysts, the mice die of cardiovascular abnormalities, revealing important extrarenal functions of the polycystins [16,17]. No mutational hot spot has been identified for either PKD1 or PKD2. In addition, the analysis of mRNA or genomic sequences of patients at risk is complicated by the duplication of most of the PKD1 gene. Hence, linkage analysis using single polynucleotide polymorphisms (SNPs) and satellite markers remains the method of choice to determine, whether an individual inherited a mutated ADPKD gene.

Nephronophthisis

Juvenile nephronophthisis (nephronophthisis type1, NPHP1) belongs to a group of genetically heterogeneous renal cystic diseases that follow an autosomal recessive mode of transmission (reviewed in [18]). Three different gene loci have been mapped, *NPHP1* (juvenile form), *NPHP2* (infantile form), and *NPHP3* (adolescent form) that differ in the onset of end-stage renal disease. *NPHP1* can be associated with extrarenal manifestations (ocular motor apraxia, retinitis pigmentosa, coloboma of the optic nerve, cerebellar vermis aplasia, liver fibrosis, cone-shaped epiphyses) (reviewed in [19]). *NPHP1*, the gene mutated in juvenile nephronophthisis has been identified [20,21]. *NPHP1* accounts for the majority of end-stage renal failure in children and young adults. The disease is characterized by tubular basement membrane disruption, interstitial cell infiltration, tubular atrophy, and cyst formation at the cortico-medullary junction [19,22]. *NPHP1* encodes for nephrocystin, a protein with 732 amino acid residues and a predicted molecular weight of 83 kD [20,21]. It contains an N-terminal coiled-coil structure, a Src-homology 3 (SH3) domain that is flanked by two highly charged domains. Recently, the crk-associated substrate $p130^{Cas}$ was found to interact with the SH3 domain of nephrocystin in yeast two-hybrid screens using either $p130^{Cas}$ or nephrocystin as a bait [18,23]. Since $p130^{Cas}$ is an essential component of focal adhesions, it has been speculated that nephrocystin regulates the assembly of focal adhesions. Targeted disruption of certain components of the focal adhesion complex results in defects that closely resemble the characteristic features of human nephronophthisis. For example, mice lacking tensin develop multiple cysts and the mice subsequently die from renal failure [24]. Mice lacking a_3b_1, an integrin, predominantly expressed in the podocyte, develop severe glomerular abnormalities, but also display cystic changes of the proximal tubules [25]. Finally, mice lacking GDIa, a GDP dissociation inhibitor of the small GTPase Rho, develop marked proteinuria as well as a degeneration of tubular epithelial cells [26]. These findings indicate that abnormalities of the focal adhesion complex may result in the development of tubular basement abnormalities in combination with renal cysts.

Tuberous Sclerosis

The tuberous sclerosis complex (TSC) is a systemic disorder, characterized by seizures, mental retardation, and benign tumors of the brain, eyes, heart, lungs, skin, and kidneys. Renal angiomyolipomas are the characteristic renal lesions, although affected individuals are also at an increased risk for renal cancer [27]. Mutations of either TSC1 (chromosome 9q34), or TSC2 (chromosome 16q13) cause tuberous sclerosis. Loss of heterozygosity in the tumors of tuberous sclerosis indicates that the TSC genes function as tumor suppressor genes (reviewed in [28]). In addition, TSC2 (rarely TSC1) mutations cause renal cysts (reviewed in [29]). The gene products of TSC1 and TSC2, hamartin and tuberin, form a protein complex that is probably mediated through their coiled-coil domains [30]. The TSC2 gene is located 63 bp upstream of PKD1, and continous gene deletions of PKD1 and TSC2 cause a particularly severe form of ADPKD with early onset renal failure [31]. Recently, a functional link was established between polycystic kidney disease and the TSC2, demonstrating that trafficking of polycystin-1 to

the basolateral membrane is disrupted in cells lacking tuberin [32]. How tuberin affects polycystin-1 trafficking remains unknown. Tuberin exerts several functions: it modulates the transcriptional activity of nuclear hormone receptors [33], affects the subcellular localization of p27 [34], and upregulates cyclin D [35]. Since tuberin accelerates the intrinsic GTPase activity of Rab5, a protein involved in endoctosis [36], it is interesting to speculate that tuberin is linked to polycystin-1 trafficking through its ability to modulate endocytosis.

Von Hippel Lindau (VHL) Syndrome

Germ-line mutations in the VHL tumor suppressor gene cause von Hippel-Lindau disease, a hereditary cancer syndrome characterized by the development of highly vascular tumors that overproduce hypoxia-inducible proteins such as vascular endothelial growth factor (VEGF). Patients develop tumors at multiple sites, including retinal angiomas, hemangioblastomas of the central nervous system, pheochromocytomas, renal cell carcinomas, and pancreatic cancers (reviewed in [37]). In some instances, VHL can present with features typical for autosomal dominant polycystic kidney disease [38,39]. The protein encoded by the VHL gene, pVHL, is part of the VCB-Cul2 complex, a close relative of the SCF family of ubiquitin-ligase complexes that links the ubiquitin-activating enzyme (E1) and ubiquitin-conjugating enzyme (E2) with the α-subunit of heat inducible factor-1 (HIF-1). Destruction of HIFα by VHL is mediated by proline hydroxylation [40,41]: above a critical intracellular oxygen tension, a specific proline of HIFα's internal oxygen-dependent degradation domain is hydroxylated by a prolyl hydroxylase. The hydroxylated proline is recognized by pVHL, which mediates the rapid degradation of HIFα through the proteasome. Hypoxia prevents the hydroxylation of HIFα; since pVHL fails to recognize the non-hydroxylated form, HIFα accumulates, interacts with its other subunit, HIFα, and binds to the HIF-response elements (5′-RCGTG-3′) in genes upregulated by hypoxia. Since the absence of pVHL and subsequent accumulation of HIF-1 facilitates the development of renal cysts, it is intruiging to speculate that hypoxia can accelerate cyst growth in polycystic kidney disease.

Conclusion

Mutations of several, apparently unrelated genes can cause renal cysts. At least two genes, PKD1 and PKD2, seem to participate in a common signaling pathway that produces calcium-permeable, non-cation selective currents. Basolateral trafficking of polycystin-1 requires tuberin, the protein encoded by one of the two tuberous sclerosis genes. Hence, cystogenesis in tuberous sclerosis appears to be linked to an altered polycystin-1/polycystin-2 function. Lack of pVHL, the protein mutated or absent in von Hippel-Lindau disease, results in HIF-1 dependent gene expression. Although it is currently unknown, whether dysregulation of HIF-1 plays a role in ADPKD, it is a well known clinical observation that dialysis patients with ADPKD tend to have higher erythropoetin and hemoglobin levels than other ESRD patients [42]. Hence, it is conceivable that local hypoxia facilitates cystogenesis. Alternatively, aberrant signaling of the polycystins in ADPKD may directly lead to dysregulation of HIF-1, a testable hypothesis that may also explain the increased risk of renal cancer in ADPKD patients.

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Interaction of the Epithelial Na^+ channel ENaC and CFTR: Crosstalk via Cl^-?

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Key Words

CFTR, epithelial Na^+ channels, ENaC, cystic fibrosis, electrolyte transport, epithelium, purinergic receptors, Cl^- channel

Abbreviations

CFTR = cystic fibrosis transmembrane conductance regulator, CF = cystic fibrosis, ENaC = epithelial Na^+ channels, $P2Y_2$ = purinergic receptor subtype.

Abstract

Background/Aims: Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) are the cause for cystic fibrosis (CF). CFTR is a cAMP activated Cl^- channel and is also affecting the function of a large number of other proteins. Previous reports demonstrated a Cl^- dependence for the inhibition of ENaC. We therefore further examined the contribution of Cl^- ions and Cl^- dependent processes on inhibition of ENaC by CFTR.
Methods: CFTR and epithelial Na^+ channels (ENaC) were expressed in *Xenopus laevis* oocytes and currents were measured 2–3 days after injection. Tracheas were isolated from mice and transepithelial voltages generated by tracheal epithelia were assessed in micro Ussing chambers.
Results: Activation of CFTR inhibited epithelial Na^+ currents expressed in *Xenopus* oocytes. This was dependent on the extracellular Cl^- concentration. ENaC was also inhibited by an increase in the cytosolic Cl^- concentration. Inhibition of ENaC by CFTR was independent of the current amplitude and amplifier used for the different experiments. The data do not indicate a CFTR mediated release of ATP.
Conclusions: CFTR is inhibiting ENaC in a Cl^- dependent manner, which is likely to be due to an increase in the intracellular Cl^- concentration in oocytes, respiratory and colonic epithelium.

Introduction

Clear evidence is now available, indicating that CFTR is a regulator of other ion conductances, apart from being a cAMP regulated Cl^- channel [1,2]. The interactions of CFTR with other ion conductances has been examined in various cell lines, *Xenopus* oocytes and native epithelial cells. From all this, inhibition of epithelial Na^+ transport by CFTR has been studied by far in most detail. Control of ENaC by CFTR seems to play a crucial role for the pathophysiology in CF airways and intestine and may therefore have a major impact on the CF phenotype. Current results have demonstrated the interaction of CFTR with various transport proteins and ion channels, such as the Na^+/H^+ exchanger type 3 (NHE3), the Cl^-/HCO_3^- exchanger, epithelial Na^+ channels, other Cl^- channels and K^+ channels [2–4]. Regulation of other membrane proteins might be the primary function of CFTR in the kidneys [5]. CFTR is expressed in most parts of the kidney tubular system and predominantly in the collecting duct. So far, there has not been any clear evidence for defective kidney function in cystic fibrosis. However, a recent study on mice homozygous for the most frequent CFTR

mutation ΔF508 indicated enhanced amiloride sensitive Na^+ absorption, which became only detectable under salt restriction [6]. Thus, kidney collecting ducts of CF patients may demonstrate enhanced ENaC conductance, similar to that present in airways and intestinal epithelium. Unlike these tissues, the CF sweat duct epithelium demonstrates reduced amiloride sensitive Na^+ conductance [7]. The underlying mechanisms for the interaction of CFTR with ENaC and other membrane proteins are still unknown, however, several possibilities have been excluded meanwhile [8,9] (Table 1). Previous studies indicated that the Cl^- transport activated through stimulation of CFTR is responsible for the inhibition of ENaC [10]. We further examined the contribution of Cl^- transport to downregulation of ENaC and found further evidence that an increase in intracellular Cl^- is inhibiting Na^+ absorption.

Methods

cRNA of rat α, β, γ ENaC and human CFTR were injected into *Xenopus laevis* oocytes. Water injected oocytes served as controls. 2 days after injection, membrane currents were measured in double electrode voltage clamp experiments. A large flowing KCl electrode served as bath reference and had a resistance of 700 Ω. Membrane currents were measured by voltage clamping of the oocytes in intervals from −90 or −50 to +20 mV in steps of 10 mV, each 1000 ms, using a OOC-1 amplifier (WPI, Germany), or a GeneClamp 500 amplifier (Axon Instruments). Mice were sacrificed, tracheas were removed, opened longitudinally and put into micro-Ussing chambers. Transepithelial voltages were assessed under open circuit conditions and short circuit currents were calculated by pulsed current injection and voltage deflections, according to Ohm's law.

Results

Inhibition of ENaC whole cell conductance was observed in *Xenopus* oocytes upon stimulation of CFTR by IBMX (1 mmol/l) and forskolin (10 μmol/l) and in the presence of high extracellular Cl^-. When most of the extracellular Cl^- was subsequently replaced by gluconate (5 Cl^-), the initial Cl^- conductance was recovered (Figure 1). Although inhibition of ENaC by CFTR in *Xenopus* oocytes has been found in numerous studies, it could not be detected in a previous report, which blamed a technical artifact being responsible for the inhibition of ENaC [11]. While the results may certainly apply to the experimental conditions chosen in this report, the data do not contradict previous results on CFTR/ENaC interaction. Unlike described in [11], we used a low conductance bath reference electrode (~700 Ω) in our previous experiments and the expressed ENaC and CFTR conductances were much lower. Thus, the contribution of the serial resistance did not exceed ~4% of the whole cell resistance [3,10]. Furthermore, inhibition of ENaC by CFTR was also observed when two bath electrodes and a virtual-ground headstage were used. Using this bath clamp, the voltage drop across the serial resistance is effectively zero (Figure 2A). Taken together, inhibition of ENaC currents following CFTR activation cannot be explained by technical artifacts. Our experiments also demonstrate that inhibition of ENaC does not depend on the amount of CFTR and ENaC coexpressed in *Xenopus* oocytes. The downregulation is observed in both oocytes expressing high or low levels of CFTR and ENaC (Figure 2B and C).

Inhibition of ENaC by CFTR was only observed when high (105 mmol/l) Cl^- was present in the extracellular bath solution. After activation of CFTR and downregulation of ENaC and when bath Cl^- was reduced to 5 mmol/l, amiloride sensitive Na^+ conductance was completely recovered (Figure 2). Interestingly, not only activation of CFTR but also purinergic stimulation of

Table 1: Potential Mechanism for the interaction of CFTR and ENaC and current evidence supporting (+) or rejecting (−) the respective mechanism

Potential Mechanism for Interaction of CFTR and ENaC	Result
• CFTR-regulated exo/endocytosis of ENaC	−
• Interaction via generation of short actin filaments	−
• Direct physical interaction	−
• Interaction via PDZ1-binding domain	−
• Nucleoside diphosphate kinase, generation of cGMP	−
• Protein kinase C, increase in intracellular Ca^{2+} Tyrosine kinase	−
• G-proteins	−
• CFTR mediated ATP/UTP release	−
• CFTR induced cell shrinkage or swelling	−
• NBF1	+
• Cl^- flux and change in intracellular Cl^- concentration	+

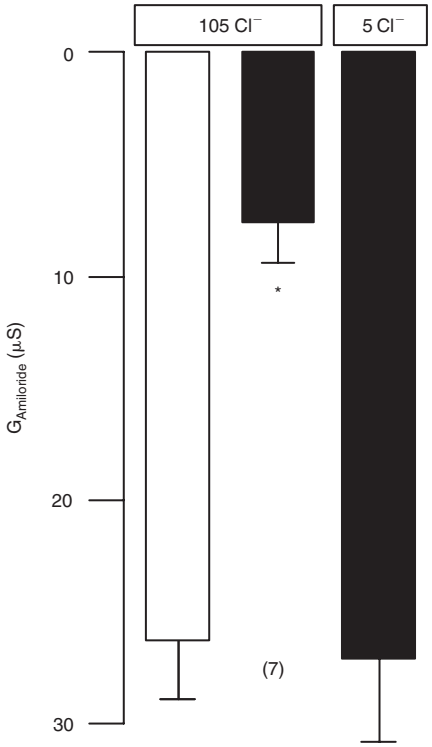

Fig. 1. Summary of experiments in *Xenopus* oocytes coexpressing CFTR and ENaC. Stimulation of CFTR in the presence of high (105mM) but not low (5mM) extracellular Cl^- inhibited amiloride sensitive ENaC Na^+ conductance. *indicate statistical significance (paired t-test, $p < 0.05$). (number of experiments).

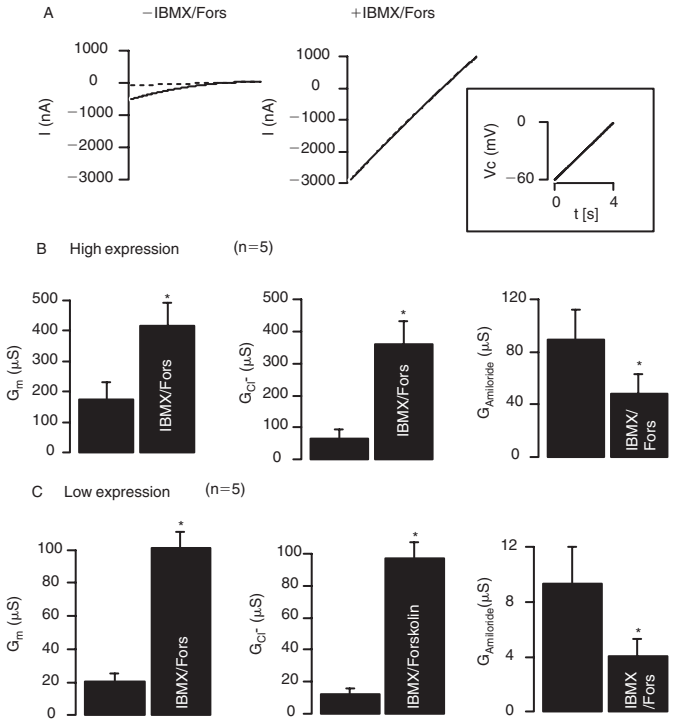

Fig. 2. A) Double electrode voltage clamp experiments in *Xenopus* oocytes coexpressing human CFTR and rat ENaC. The experiments were performed with a GeneClamp 500 amplifier (Axon Instruments) and two bath electrodes with virtual-ground headstage. Measurement of whole cell currents using a voltage ramp protocol (−60 to 0 mV within 4 s; inset) before (left trace) and after (right trace) stimulation with IBMX (1 μmol/l) and forskolin (10 μmol/l). Currents were obtained in the absence (solid line) and presence (dotted line) of amiloride. No amiloride-effect was detectable after stimulation with IBMX and forskolin. Summary of experiments from oocytes expressing high (B) or low (C) levels of CFTR and ENaC. Note that inhibition of ENaC during activation of CFTR by IBMX (1mmol/l) and forskolin (10 μmol/l) is observed in both batches of oocytes.

airway epithelial cells inhibits amiloride sensitive Na^+ transport [8]. Because previous studies have demonstrated that CFTR facilitates autocrine ATP secretion [12], we examined a potential mechanism by which activation of CFTR releases ATP to the luminal side of epithelial cells, which then inhibits Na^+ conductance (Figure 3). In order to test for this hypothesis, experiments were performed in mouse trachea and in *Xenopus* oocytes coexpressing CFTR, purinergic $P2Y_2$ receptors, ENaC and endogenous Ca^{2+} activated Cl^- channels (CaCC). However, using the $P2Y_2$ receptor inhibitor suramin, the blocker of Ca^{2+} activated Cl^- channels DIDS and the ATP hydrolyzing enzyme hexokinase, we were unable to detect a CFTR mediated release of ATP and inhibition of ENaC.

Because Cl^- is required for inhibition of ENaC, we examined the effect of Cl^- ions on ENaC conductance expressed in *Xenopus* oocytes. A limited nonselective cation/anion permeability was induced by partial permeabilization using low (1.2 μg/ml) concentrations of amphotericin B. Thus, a rapid adaptation of the intracellular ion concentration to that of the bath solution was expected [13]. In the absence of amphotericin B,

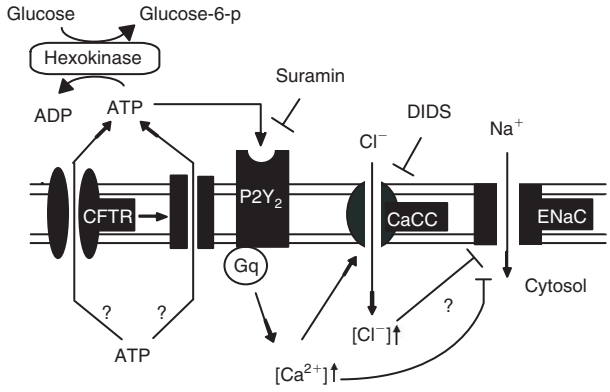

Fig. 3. Model for CFTR mediated ATP release in *Xenopus* oocytes or airway epithelial cells coexpressing CFTR, ENaC, purinergic $P2Y_2$ receptors and $Ca2^+$ activated Cl^- channels. Stimulation of CFTR leads to secretion of ATP by CFTR or by an independent ATP channel. ATP will bind to $P2Y_2$ receptors, thereby activating Cl^- transport. Activation of $P2Y_2$ receptors lead to inhibition of epithelial Na^+ channels (ENaC).

we changed to a bath solution containing only 5 mM Cl^- and 20 mM Na^+. Partial permeabilization by amphotericin B in the presence of low extracellular Cl^- did not affect ENaC conductances. Subsequent increase of Cl^- from 5 to 50 mM largely attenuated ENaC conductance from 19.4 ± 3.4 to 5.3 ± 1.2 μS. Moreover, when the effects of various concentrations of extracellular Cl^- on amiloride sensitive Na^+ conductance were examined, we found a 50% inhibition of ENaC at about 25 mmol/l and 80% at 50 mmol/l. Upon returning to 5 mM bath Cl^-, about 50% of the initial ENaC conductance was recovered. These data suggest regulation of ENaC by intracellular Cl^- concentration in *Xenopus* oocytes, similar to what was been described for mandibular duct cells [14]. Thus, Na^+ transport in airway epithelial cells could be inhibited by CFTR mediated by activation of a Cl^- conductance and increase in the cytosolic Cl^- concentration.

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Leak Potassium Channels with Two Pore Domains

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Key Words

Background K^+ channels

The Leak Channel Gene Family

All cells exhibit a resting electrical potential across the plasma membrane. This resting potential (E_r) corresponds mainly to the diffusion potential of K^+ ions through the membrane. A high concentration of K^+ is created inside the cell by active pumping, and the outward passive diffusion of these ions through K^+ selective channels generates the inside-negative transmembrane potential. Whereas any type of K^+ channel that opens at rest may be involved in the generation and maintenance of E_r, it is now recognized that a special class of K^+ channels is devoted to this task [1]. At the macroscopic level, the activity of these channels seems to be almost time- and voltage-independent. Thus they are generally called leak, baseline, or background channels. Another characteristic of the leak channels is their weak sensitivity to classical blockers of K^+ channels, such as TEA, 4-AP and Cs^+. Since the original cloning of TWIK1 in 1996, 14 related genes have been finally isolated from human (Figure 1) (for review see [1] and for the most recently cloned channels [2–7]). Such a large number of genes is surprising given the apparent basic function of these channels. Data gathered from genome sequencing projects reveal that this channel family is old and widely distributed in the animal kingdom. More than 40 related genes have been identified in the nematode *Caenorhabditis elegans* and 12 in the fruit fly *Drosophila*. Compared to the human channels, the study of these nematode and fly channels is still in its infancy and, except for two of them, no functional data are yet available [8,9].

Protein Structure of Leak Channels

All the TWIK1-related proteins are 300 to 500 residues long and share similar hydropathic profiles predicting four transmembrane segments. Perhaps the most salient feature is the presence of two pore-forming (P) domains in the channel sequence. P domains are crucial for the formation of the pore selectivity filter. Many K^+ channels have only one P domain. Given that K^+ channels with one P domain are active as tetramers, it has been hypothesized early that leak channels with two P domains were active as dimers. As expected, TWIK1 forms dimers and these dimers contain an interchain disulfide bridge [10]. The cysteine residue involved in this bond is part of the extracellular loop located between the first transmembrane segment (M1) and the first P domain (P1). The predicted structure of this M1P1 loop is an alpha helix containing a regular occurrence of hydrophobic and charged residues. This profile is typical of interdigitating helices that interact through hydrophobic interactions. Figure 2A shows an alignment of these loops in different channels. The regular occurrence of hydrophobic and charged residues is conserved, as is the cysteine involved in the covalent bridging. TASK1 does not possess this residue and, as shown in Figure 2B, is unable to form covalent dimers compared to the other channels. However, a functional approach has recently demonstrated that this channel was also active as a dimer [11]. In addition, the covalent dimerization of some of these channels has been confirmed by Western blot analysis of native proteins [12–14]. These results support the idea that both P domains are functional and are involved in the formation of the ionic pore. In the leak channels, the first P1 domain can accommodate residues that are never observed in the one-P channels and that can suppress the channel activity when introduced in these channels [1]. The unusual symmetry resulting from dimerization (P1-P2-P1-P2)

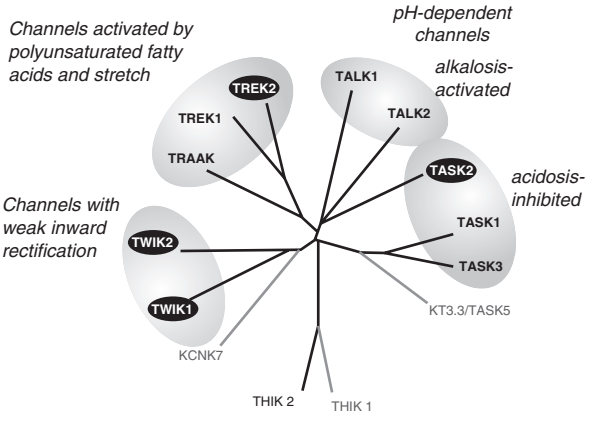

Fig. 1. The leak two P domain K^+ channel family. The dendrogram of human proteins has been established by using ClustalW and Treeview. The different functional subclasses are indicated as well as the silent subunits (light gray). THIK2 is a functional leak channel that does not belong to any particular subclass. The most abundant leak channels in human kidney, TWIK1, TWIK2, TASK2 and TREK2, are highlighted.

probably provides an evolutionary flexibility that is not possible with the symmetry of tetramers in one P channels (P-P-P-P). Two-P domain channels are active as homomultimers, and there is no evidence that these channels form heteromultimers, as it has been documented for many one-P domain K^+ channels.

Functional Properties of Leak Channels

When expressed in *Xenopus* oocytes and in mammalian cells, cloned two-P domain K^+ domain channels strongly polarize the resting membrane potential, as expected for leak K^+ channels active at rest. The currents they mediate are very selective for K^+ ions, display quasi-instantaneous activation and inactivation kinetics and follow the K^+ electrochemical gradient. At first glance, leak channels can be considered K^+-selective holes in the membrane and could appear to behave simply when compared to more sophisticated channels such as voltage-gated or Ca^{2+}-sensitive K^+ channels. In physiological conditions, when K^+ concentration is higher in the cell than outside, leak channels carry more outward current than inward current. This outward or "Goldman-Hodgkin-Katz" rectification is not related to any voltage-sensor as described for voltage-gated K^+ channels, but is simply a consequence of the laws of diffusion. As predicted by the constant-field current equation, the current-potential relationships for the leak currents become linear when K^+ is equally distributed across the cell membrane. TASK1 possesses all the characteristics of such archetypal leak channel behavior. It produces time- and voltage–independent currents that are not sensitive to the classical blockers of K^+ channels 4-AP, TEA, and Cs^+, and that display no rectification other than the one predicted by the diffusion laws. However, the electrophysiological properties of some other two-P domain K^+ channels slightly diverge from this "ideal" behavior. Some channels are not strictly time-independent and possess very fast but discernable kinetics of activation as TASK2, or inactivation as TWIK2. Some others are not strictly voltage-independent, as the TWIK channels, which are inwardly-rectifying in physiological conditions, and TREK1 which remains outwardly-rectifying even when the K^+ concentrations are identical across the cell membrane. From a pharmacological point of view, some of these channels are sensitive to quinine and quinidine, and to Ba^{2+}, albeit at high concentrations. These singularities as well as their unique single channel conductances ranging from less than 5 to 110 pS can be used to identify each channel type in transfected and native cells (for a detailed review of these electrophysiological properties see [15]).

As observed in the other K^+ channel families with one P domain and six or two transmembrane domains, some two-P domain proteins do not form active channels. Out of 14 channels, three members of this family do not produce K^+ currents when expressed in *Xenopus* oocytes or transfected cells. These silent subunits could require additional subunits to produce functional channels, or may correspond to intracellular channels [1,6,7].

Regulatory Properties of Leak Channels

A fascinating aspect concerning the leak K^+ channels with two P domains is the extreme variety of mechanisms for regulating channel activity (reviewed in [1,15]). Based on these specific modulations, several subfamilies of two-P domain K^+ channels can be distinguished. This functional classification roughly correlates with the sequence conservation between the proteins, TASK2 being the most evident exception (Figure 1).

TREK and TRAAK channels form a subfamily of channels activated by unsaturated fatty acids and lysophospholipids. In addition, they are strongly stimulated by increasing the mechanical pressure applied to the cell membrane and they are closed by hypoosmolarity. TREK1 is strongly upregulated by temperature. As expected for mechano-gated channels, TREK1, TREK2 and TRAAK are blocked by $Gd^{3+}$and amiloride. They are also sensitive to a modification of the intracellular pH. Acidosis stimulates TREK1 and TREK2, while alkalosis potentiates TRAAK. In general, both intracellular and extracellular pH shifts have strong effect on the two-P domain K^+ channels. Two additional subclasses of channels are recognized according to their pH-sensitivity: the TASK channels, which are inhibited by external acidosis in the physiological range, and the TALK channels, which are activated by alkalinization of the external medium. TWIK channels are downregulated by intracellular acidification. Other chemicals that have effects on two-P domain K^+ channels are the endocannabinoid anandamine that specifically blocks TASK1 [16], and some volatile general anesthetics that stimulate TREK and TASK channels and inhibit THIK1 and TWIK2 [6,17–19]. In addition to sensitivity to a variety a chemical and physical stimuli, the two-P domain K^+ channels are also modulated via G-protein-coupled receptors and phosphorylation /dephosphorylation mechanisms [1,15]. For example, stimulation of the Gs-coupled receptors 5HT4sR or the Gq-coupled

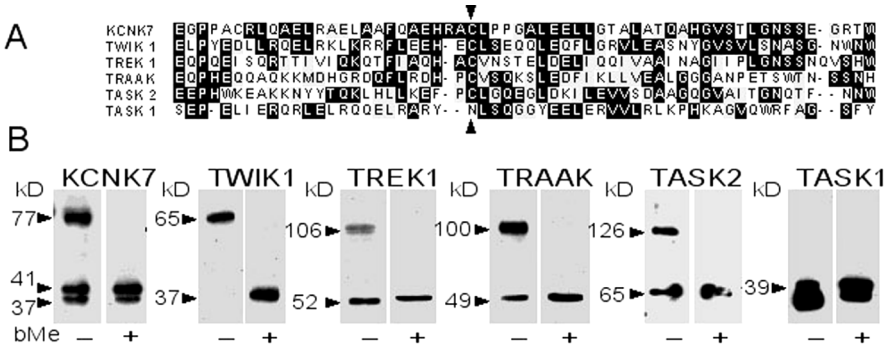

Fig. 2. Two P domain K^+ channels are homodimers. A, Sequence alignment of M1P1 loops. The cysteine involved in the disulfide-bridged dimerization of TWIK1 and conserved in the other proteins is indicated by arrows. B, Western blot of proteins from recombinant baculovirus-infected Sf9 cells analyzed in the presence or absence of the reducing agent β-mercaptoethanol (β-ME). Channel proteins are detected by using specific or anti-tag antibodies.

receptor mGluR1 inhibits TREK2, whereas activation of the Gi-coupled receptor mGluR2 increases TREK2 activity [5]. The effect of 5HT is mimicked by a membrane-permeant derivative of cAMP. In the case of TREK1, the phosphorylation by protein kinase A is responsible for the closing of the channel. Like TREK2, TASK1 is inhibited by activation of receptors coupled to Gq and in both cases, neither Ca^{2+}nor protein kinase C seem to be involved in the effect.

Leak Two-P Domain K^+ Channels in Native Tissues

In contrast to the other types of K^+ channels that were first described and characterized in native tissues and cells before being cloned, leak K^+ channels and their properties were mainly studied after cloning and expression in heterologous systems. The number of studies concerning native background K^+ channels was limited because of the leak characteristics and weak amplitudes of these currents as well as the absence of specific pharmacology. The cloning and characterization of the two-P domain K^+ channels has strongly stimulated this field, and studies dealing with the physiological roles of these channels are now emerging at a high pace.

The most studied channel is TASK1. In the nervous system, this channel (perhaps together with TASK3 in some cases) has been shown to be important for the control of cerebellar granule cell and motoneuron excitability (see review by [20]). TASK1 is active at rest and its closure by neurotransmitter is associated with a depolarization of the cell membrane and increase in cell excitability. In KO mice lacking the inhibitory conductance mediated by the $GABA_A$ receptor, the response of cerebellar granule cells to excitatory synaptic input remains unaltered, owing to the increase in a TASK-like leak conductance [21]. The opening of TASK channels by general anesthetics, together with TREK1 and 2, is expected to contribute to the depressing effects of general anesthetic in the central nervous system [17–19]. More specifically, TASK1-activation in motoneurons would contribute to anesthetic-induced immobilization whereas in locus coerelus neurons, it may support analgesic and hypnotic actions attributed to inhibition of these cells [18]. TREK channels are expected to play roles similar to TASK1. But given that they share many functional characteristics with the S-type channels of *Aplysia*, they may be more specifically involved in presynaptic modulation and thus in learning as demonstrated for the S channels [5,15]. On the other hand, the temperature sensitivity of TREK1 suggests that it could play a role of cold sensor in the thermosensitive neurons that express it [13].

In the cardiovascular system, TASK1 is expressed in chemosensitive carotid body cells, where its closing upon acidosis and hypoxia induces the depolarization initiating dopamine release and ultimately the reflex respiratory increase [22]. A role for TASK1 has also been proposed for the generation of a high resting potential in glomerulosa cells of the adrenal gland. Again, its closure upon angiotensin II receptor activation triggers the depolarization-activated Ca^{2+} entry and aldosterone release [23].

At least four leak channel genes show a relatively high expression in the kidney (Figure 1). This assessment mainly relies on Northern blot and RT-PCR experiments [1,24], and our knowledge of the precise protein localization is very poor. Up to now, they are no *in situ* hybridization or immunohistochemical studies that report the kidney distribution for TWIK2 and TREK2. In mouse kidney, both TWIK1 and TASK2 are present in the proximal tubule, with the highest expression in the S3 segment (unpublished data). The intracellular distribution of TASK2 is not yet known. Interestingly, TWIK1 is located in the subapical rim of the vacuolar apparatus, under the brush border of proximal tubule cells (Dr. Brigitte Kaissling, personal communication). The exact function of TWIK1 and TASK2 in this nephron segment is still not understood. Knock-out mice for these two channel genes are now available and will help to analyze their physiological functions.

Clearly, our knowledge of physiological and pathophysiological roles of the leak two-P domain K^+ channels remains very limited and concerns mainly the nervous system. In many organs where they are expressed, their roles are yet unknown. Whether the modulations of these channels *in vitro* by pH, fatty acids and membrane stretch have physiological relevance remains to be explored. There is no doubt that the development of KO mice as well as the identification of specific pharmacological agents represent two important steps toward the detailed study of these channels. Finally, it is also important to note that these leak channels with two pore domains represent a potentially interesting target for the development of pharmaceutical drugs.

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Role of Ion Channels in Endothelial Vasoregulation

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Abstract

Ion channels play a pivotal role in vascular endothelial cell function by controlling Ca^{2+}-influx and cell hyperpolarization. For instance flow-induced vasodilation as well as Ca^{2+}-dependent NO production can be completely abolished by ion channel blockers. This article focuses on different types of ion channels in the endothelial cell membrane and how they control endothelial function with respect to Ca^{2+} signaling, mechano-sensor function and cell membrane potential. An overall principal function of ion channels in the endothelium is the control of Ca^{2+} homeostasis either directly by mediating Ca^{2+} entry or indirectly by controlling the membrane potential, which provides the electrochemical driving force for Ca^{2+} influx. We describe two general types of non-selective cation channels which mediate sustained Ca^{2+} influx after receptor stimulation or which are sensitive to mechanical stimulation of the endothelium and induce Ca^{2+} influx more directly. We also describe Ca^{2+}-activated K^+ channels and their importance in the regulation of endothelial membrane potential as well as their role in controlling endothelium-dependent hyperpolarization of vascular smooth muscle and relaxation.

Key Words

Endothelium, calcium signaling, mechanosensitive channels, store-operated channels, Ca^{2+}-activated potassium channels

Introduction

The endothelium is considered a highly differentiated signal-transduction interface as it converts humoral stimuli into biochemical signals. It plays an important role in immunological processes, thrombocyte aggregation, barrier function, and especially in the control of the contractile state of the vascular smooth muscle. In addition, the endothelium triggers angiogenesis and vessel repair after injury. Such multiple functions are mediated by the synthesis of a variety of factors that influence the function of blood cells, vascular smooth muscle cells as well as the function of the endothelium itself. The most known factors controlling vascular tone are nitric oxide (NO), prostaglandines, endothelins, and the endothelium-derived hyperpolarizing factor [1,2,3]. These factors have been proposed to be not only synthesized after receptor stimulation e.g. by circulating small vasoactive petides such as bradykinin, or by acetycholine and ATP, but also by alterations in the mechanical stress elicited by changes in the blood flow rate or the intravascular pressure [1,2]. In the underlying signal transduction mechanisms, Ca^{2+} mobilization from intracellular stores and Ca^{2+} influx have been considered one of the key steps since the synthesis of vasoactive factors like NO, prostacyclin, EDHF is Ca^{2+} dependent [4,5,6]. Especially, the activation of Ca^{2+}-permeable cation channels in response to agonists or changes in hemodynamic environment may represent a pathway for Ca^{2+} influx. Although some investigators reported the presents of voltage-gated Ca^{2+} channels (for review, see 6), ECs are generally considered as electrically non-excitable cells and it is well accepted that voltage-gated Ca^{2+} channels do not play a functional role in Ca^{2+}-signaling in the majority of ECs.
The endothelial membrane potential exerts an important control in Ca^{2+} entry by providing the sufficient electrochemical driving force [6,7,8]. The membrane potential is supposed to be mainly controlled by the activity of K^+ channels. However, also Cl^- channels and non-selective cation channels may significantly influence the membrane potential [6]. With respect to K^+ channels, recent studies suggest that Ca^{2+}-activated K^+ channels mediate membrane hyperpolarization which could be electrotonically transmitted via myoendothelial gap junctions to the vascular smooth muscle and could therefore control vascular tone independently from endothelial-derived vasoactive substances [9,10].
Many studies on endothelial ion channels are hampered by the investigating ion channel function in cultured EC. These isolated EC kept under artificial and static cell culture conditions for several passages might not necessarily reflect the electrical properties of EC in the intact vessel *in vivo*. Moreover, by investigating EC from various species and originated from distinct vascular beds, insights into endothelial ion channel function is still limited, in particular regarding ion channels in human arterial endothelium [11]. Here we describe mechanosensitive cation channels and receptor-regulated cation channels as well as Ca^{2+}-activated K^+ channels which have been identified in intact vessel preparations of human mesenteric artery [11,12] and in intact macro- and microvascular endothelium of the rat [13–16].

Mechanosensitive Cation Channel in the Endothelium

The endothelium is exposed permanently to hemodynamic forces such as the viscous drag or shear stress elicited by the streaming blood and the biaxial stretch resulting from changes in the transmural pressure. Such forces stimulate the endothelium and induces signal transduction events in which activation of mechanosensitive channels (MSC) seem to be involved [5,17].

It has been proposed that these mechanosensitive channels act as microtransducers sensing mechanical stimuli and convert them into an intracellular signal, i.e. an increase in intracellular Ca^{2+} concentration by a Ca^{2+} influx. The exact mechanism of channel activation is still elusive. Cytoskeletal structures and focal adhesions have been proposed to transduce mechanical stress to the channel protein [18]. In addition, mechanical activation of tyrosine kinases might induce channel phosphorylation and thus channel opening [6]. A well known class of mechanosensitive channels are stretch-activated cation channels (SAC) which have been identified in EC from several species such as in porcine aortic EC [17], rat aortic EC [14], and in endothelium of human umbilical vein (16) and renal arteries (Figure 1). Single channel conductance of these SAC range between 30–50 pS for monovalent cations and 10–19 pS for Ca^{2+}. In single channel recordings, these SAC can be gradually activated by increasing membrane stretch (Figure 1) and permit a sufficient Ca^{2+} influx to increase intracellular Ca^{2+} concentration. Measurements in intact endothelium of aorta and mesenteric arteries of the rat made it possible to identify another class of mechanosensitive cation channels [13] which exhibit a mechanosensitivity distinct from SAC. The PAC are inactivated by membrane stretch and solely activated by applying positive pipette pressure to the cell membrane (Figure 2). This pressure-activated channel (PAC) has a lower single channel conductance for monovalent cation (20–25 pS) and is also permeable for Ca^{2+} (5–6 pS). For both SAC and PAC, it has been shown that in the presence of physiological Ca^{2+} concentrations Ca^{2+} entry through the channels is sufficient to increase intracellular Ca^{2+} concentration [13,15,19]. The function of these channel has also been demonstrated in whole-cell experiments in which whole-cell currents through MSC were activated by osmotic stress induced changes in membrane tension [6,13]. MSC also triggers shear stress-induced oscillations of intracellular Ca^{2+} concentration and membrane potential in bovine endothelial cells [20]. In addition to mechanosensitive non-selective cation channels, cell-swelling activates volume-activated Cl^- channels and K^+ channels [6] which presumably are important in regulating cell volume.

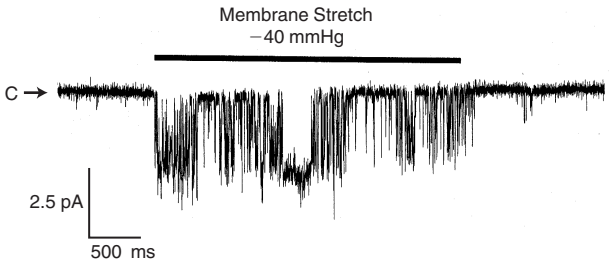

Fig. 1. Original current recording of a 30 pS stretch-activated cation channel in intact endothelium of kidney artery. In the cell-attached patch-clamp configuration, downward-depicted currents indicate Na+-influx into the cell at holding potential of −80 mV. C, denotes closed state of the channel.

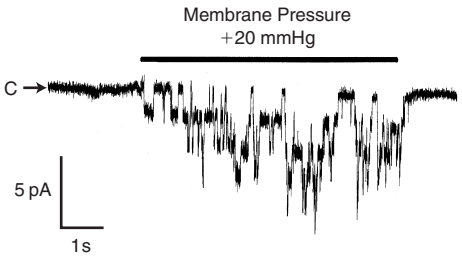

Fig. 2. Original current recording of a 22 pS pressure-activated cation channel in intact endothelium of the rat. In the cell-attached patch-clamp configuration, downward-depicted currents indicate Na+-influx into the cell at holding potential of −80 mV. C, denotes closed state of the channel. Superimposed current amplitudes indicate up to 4 channels simultaneously activated by applying positive pressure to the cell membrane.

Endothelial Receptor-regulated Non-selective Cation Channels

Stimulation of the endothelium with circulating factors such as bradykinin and histamine, which bind to G-protein coupled receptors, induces a rapid and transient Ca^{2+} release from $InsP_3$-sensitive stores which is of crucial importance for the Ca^{2+}-dependent synthesis of vasodilating factors. The initial Ca^{2+} peak is followed by a sustained Ca^{2+} plateau or Ca^{2+} oscillations which depend on Ca^{2+} influx into the endothelial cell. The putative channel mediating this Ca^{2+} entry subsequent to store depletion has been named SOC (store-operated Ca^{2+} channels or CRAC (Ca^{2+}-release-activated Ca^{2+} channel). As potential genes encoding for such an SOC, mammalian homologues of the transient receptor potential (*trp*) gene have been implicated [21–25]. All functionally expressed TRP genes (TRP1–10) code for cation non-selective Ca^{2+} permeable channels. However, it is still a matter of controversy whether these TRP channel can be classified as store-operated or receptor-second messenger regulated channels since e.g. TRP3 and TRP6 appear to be insensitive to store depletion, but are directly activated by diacylglycerol [25], whereas TRP4 has been classified as store-operated [25]. Expression of some TRP channels has been detected, although inconsistently so, in cultured endothelial cells (EC) [26–29]. For instance, a recent study showed expression of the TRP genes TRP1, TRP3 and TRP4 gene in cultured human umbilical vein endothelial cells [26]. Another study showed expression of TRP1 but not of TRP3 and TRP6 in cultured human pulmonary artery endothelial cells [27]. However, a single-cell RT-PCR study on human mesenteric endothelial cells (HMAEC) *in situ*, revealed only expression of TRP1 and TRP3 [12]. Moreover, TRP1 appeared to be the predominantly expressed member of the TRP genes in endothelium of human mesenteric artery which is consistent with the apparently wide tissue distribution of this gene [21] whereas expression of TRP3, TRP4, and TRP6 has been suggested to be more restricted to the brain. These discrepancy between expression of TRP genes in endothelium *in situ* and that found in isolated and cultured human EC could be due to alterations of expression of TRP channels as a consequence of cell isolation and highly artificial cell culture conditions. Therefore, differences in channel expression *in situ* and in cell culture suggests that ion channel expression in cultured EC does not necessarily reflect the original *in vivo* expression pattern.

In patch clamp experiments in HMAEC *in situ*, activation of single cation channels was detected after agonist stimulation and activation of whole-cell cation currents by infusion of $InsP_3$ or Ca^{2+}. The non-selective Ca^{2+}-permeable cation channel observed in single channel patch-clamp experiments is characterized by well distinguishable channel openings and had a unitary conductance of 26 pS for monovalent cations. This channel matches some electrophysiological properties of a human TRP1

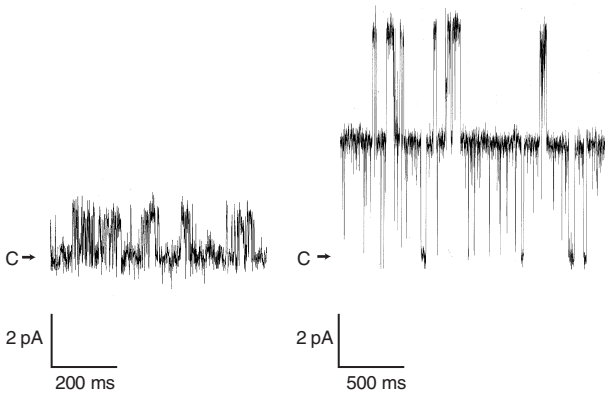

Fig. 3. Original current recording of intermediate conductance Ca^{2+}-activated potassium channel (left trace) and large-conductance Ca^{2+}-activated potassium channels (right trace with two simultaneously active channels) in excised inside-out patches at holding potential of +90 mV and 0 mV, respectively, and in symmetrical 140 mmol/L K1 solution and 400 nmol/L cytosolic $[Ca^{2+}]$free. Upward depicted channel currents represent K1 efflux. C, denotes closed state of the channel.

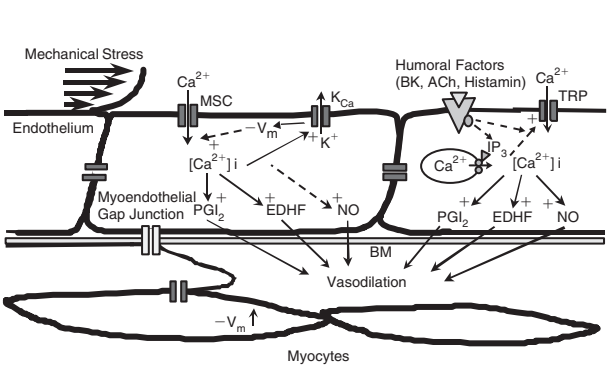

Fig. 4. Schematic illustration of the role of ion channels in endothelial function.

functionally expressed in *COS* cells [21,30]. TRP3 is characterized by very short lasting openings [24]. However, such TRP3 related single-channel currents have not been detected in HMAEC or cultured EC so far [12,29]. Interestingly, with respect to the activation mechanism for TRP3, increases of the cytoplasmic Ca^{2+}-concentration and diacylglycerol have been shown to activate TRP3 channels functionally expressed in *COS* cells [24]. In whole-cell patch-clamp experiments, increases of the intracellular Ca^{2+} concentration activated non-selective cation currents. This might indicate that TRP3 channels, presumably as part of heteromultimeric channels, contributes to the Ca^{2+}-activated non-selective cation current in ECs. In conclusion, there is good evidence that Ca^{2+} entry through TRP1/3 channels might represent important components of endothelial Ca^{2+} signaling and thereby of endothelial function in human blood vessels.

Endothelial Ca^{2+}-activated K^+ Channels

Ca^{2+}-activated K^+ channels (K_{Ca}) are important regulators of endothelial function. Blockade of the K_{Ca} with highly specific channel blockers like charybdotoxin or iberotoxin can completely abolish the endothelial dependent vasoldilation or NO production [8,31].

Ca^{2+}-activated K^+ channels mediate endothelial hyperpolarization in response to humoral stimulation. This endothelial hyperpolarization provides the electrochemical driving force for Ca^{2+} entry which promotes adequate Ca^{2+}-dependent synthesis of vasodilators. Moreover, recent studies demonstrated that endothelial hyperpolarization might directly induce vasodilation. The endothelial hyperpolarization is propagated via gap-junctional coupling to the underlying vascular smooth muscle cells (VSMC) and thereby leads to the closure of voltage-gated Ca^{2+}-channels in VSMC [10]. Also, K^+ efflux through endothelial K_{Ca} has been shown to induce relaxation of VSMC by stimulating inwardly rectifying K channels in VSMCs and was therefore proposed to serve as an EDHF [9]. Blocking of endothelial hyperpolarization with K_{Ca} selective inhibitors such as apamin and CTX prevents such an EDHF-mediated vasodilation thus indicating that activation of endothelial K_{Ca} is essential for non-NO and non-prostacyclin-mediated vasodilation. Based on single channel conductance and toxin-sensitivity, three classes of K_{Ca} have been identified in EC [6,11,16,32,33]: an apamin-sensitive small-conductance K_{Ca} (SK_{Ca}) with a single-channel conductance of 8–10 pS, a charybdotoxin (CTX)-sensitive intermediate-conductance K_{Ca} (IK_{Ca}) with a single-channel conductance of 25–40 pS, and a CTX and iberiotoxin (IBTX) sensitive large-conductance K_{Ca} (BK_{Ca} or Maxi K) with a single channel conductance of 150–300 pS. SK_{Ca} and IK_{Ca} are characterized by a high Ca^{2+}-sensitivity with an EC_{50} in the nanomolar range (~300 nmol/L), voltage-independence, and slight inward-rectification. BK_{Ca} are less Ca^{2+}-sensitive with an EC_{50} in the micromolar range (~3 μmol/L), non-rectifying, and channel activity is highly voltage-dependent. Single-channel currents of SK_{Ca} and IK_{Ca} have been detected in intact endothelium of rat aorta [32]. SK_{Ca} and IK_{Ca} mediated whole-cell currents were observed in electrically uncoupled EC of rat carotid arteries [34], where they mediate endothelial hyperpolarization after agonist stimulation. With respect to human ECs, IK_{Ca} is the predominant K_{Ca} in intact endothelium of mesenteric arteries, whereas SK_{Ca} and BK_{Ca} play only a minor or no role in these vessels. In HUVEC, all three types of K_{Ca} have been described [6], although IK_{Ca} is presumably of major importance compared to the other two. In freshly isolated HUVEC, bovine aortic endothelial cells, and in intact human endothelium of mesenteric arteries, K_{Ca} currents are almost completely blocked by CTX or clotrimazole, a more selective blocker of IK_{Ca}. In contrast, apamin and IBTX do not have considerable blocking effects [11]. In porcine endocardial endothelium and aortic ECs, mainly BK_{Ca} and IK_{Ca} confer K_{Ca} currents [19]. The type of endothelial K_{Ca} being involved may differ substantially between species and the vascular beds investigated [11,19,32–34].

Single-cell reverse transcription - polymerase chain reaction *in situ* of human mesenteric arteries [11] and rat arteries [34] revealed that these ECs express the IK1 and SK3 gene whereas expression of the BK_{Ca} gene Slo, coding for the pore-forming α-subunit, was detected much less frequently.

Regarding the functional role of endothelial K_{Ca} activation in EDHF-mediated relaxation, it has been shown that blocking of K_{Ca} by the combination of apamin and CTX completely suppressed non-NO and non-prostacyclin mediated relaxations [9]. Thus this indicates that SK_{Ca} and IK_{Ca} are the major players in the initial generation of the EDHF response.

Endothelial Ion Channel Function in Hypertension

There is growing evidence that vascular ion channel function is altered in cardiovascular disease states like hypertension. Although ion channel function has been extensively investigated vascular smooth muscle cells from rat models of hypertension and mice-knockout models [35–38], much less is know about endothelial ion channel function in hypertension. In particular, it has been proposed that the disturbed endothelium-dependent flow-induced vasodilation present in hypertension [39] might be due to disturbed function of mechanosensitive channels in the endothelium. In the SHR-model of genetic spontaneous hypertension and in 2K1C renovascular hypertensive rats MSC function was increased [13] which was interpreted as a compensatory and protective mechanism to increase Ca^{2+}-entry in the presence of increased hemodynamic forces and thereby to improve Ca^{2+}-dependent synthesis of vasodilators in the presence of elevated blood pressure. Such alterations in MSC function were reversed after effective antihypertensive therapy [40]. However, in genetic salt-sensitive hypertension (Sabra) and SHRSP, a defective regulation of MSC was observed with an abolished protective upregulation of the channel density in hypertension [40,41] which leads to a lowered mechanosensitive Ca^{2+} influx and a consequently deminished synthesis of vasodilating factors. Thereby in experimental hypertension the dysregulation of mechanosensitive channels in resistance arteries contributes to the endothelial dysfunction, especially with respect to flow-induced endothelium-dependent vasodilation.

Whether the function of endothelial K_{Ca} or other non-selective cation channels such as TRP is altered in hypertension, is still elusive and, in this regard, future studies focusing on K_{Ca} and TRPs might bring new insights into the understanding of the defective endothelial signal transduction in hypertension.

Conclusions

Important determinants of endothelial function are the intracellular Ca^{2+} concentration regulating the Ca^{2+} dependent synthesis of NO, prostacyclin, or EDHF and the cell membrane potential. Both are regulated by a set of specialized endothelial ion channels. The Ca^{2+} influx occurs through Ca^{2+} permeable non-selective cation channels and mechanosensitive cation channels which are either activated by humoral substances via specific receptors or by hemodynamic forces. The Ca^{2+} influx itself would be limited by its own depolarizing effect unless the negative membrane potential is maintained by hyperpolarizing cation efflux. This is brought about by Ca^{2+}-activated K^+ channels which are precisely tuned by the intracellular Ca^{2+} concentration or influx. Recent studies suggest that these Ca^{2+}-activated K^+ channels are part of the EDHF-induced vasodilation. Apparently endothelial hyperpolarization by the K^+ channels is transmitted via myoendothelial junctions to the underlying vascular smooth muscle cells.

The observation that endothelial vasodilation can be completely blocked by specific ion channel blockers demonstrates the importance ion channels in endothelial function. In hypertension ion channels could contribute to the endothelial dysfunction which leads to an elevated peripheral resistance. Especially the shear stress-dependent endothelial dysfunction may be based on an impaired regulation of mechanosensitive Ca^{2+} permeable cation channels in resistance vessels which leads to a decreased Ca^{2+} infux and decreased synthesis of vasodilators.

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Chemokines and Inflammatory Renal Diseases

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Key Words

Monocyte chemotactic peptide-1, vMIP-II, triple-helix forming oligonucleotides, chemokine antagonist, inflammation

Introduction

The multi-step process of leukocyte migration from the periphery into tissues is a hallmark of inflammation. Central events in this process are mediated by members of the large chemokine family. Chemokines are a family of rather small, chemotactic cytokines that were initially subdivided into two main branches, according to the position of conserved cysteines [1,2]. Most of the known chemokines belong to the CXC and CC subfamilies, and two additional branches each containing only one member (C and CX3C chemokines) were introduced more recently. These proteins mediate their specific effects through seven transmembrane domain G protein-coupled receptors [3]. So far, six receptors for CXC chemokines (CXCR1 to 6), eleven receptors for CC chemokines (CCR1 to 11), one CX3C chemokine receptor (CX3CR1), and one C chemokine receptor (XCR1) have been identified [4]. Most receptors recognize more than one chemokine, and several chemokines bind to more than one receptor, indicating redundancy of the chemokine system. In addition to their role in mediating leukocyte migration, a number of chemokine receptors were found to be required by HIV as coreceptors for cell entry [5].

The Role of Chemokines in the Course of Renal Inflammation

The expression of chemokines by renal cells and their contribution to kidney inflammation has been well documented during the last decade [6,7]. Renal infiltration of target cells such as monocytes/macrophages and T lymphocytes is a critical event in kidney inflammation, and this complex and dynamic process is largely controlled by chemokines. So far, therapeutic strategies for the treatment of renal diseases have been limited to steroids blocking inflammatory gene expression or immune suppressants and cytostatic drugs for general ablation of proliferative immune responses. Newly developed receptor-antagonizing compounds have only been tested in experimental models of nephritis [6,7]. In general these studies have led to the expected result, i.e. immune mediated kidney injury was diminished. However, both the extend of anti-inflammatory potency of single chemokine blockade and some conflicting data showing even pro-inflammatory effects of chemokine antagonists raised doubt concerning the clinical applicability of this anti-inflammatory strategy. Also with the number of chemokines differentially expressed in the healthy as well as injured kidney still increasing, the inter play of housekeeping migratory and regulatory roles of chemokines, such as IP-10 or MCP-1, requires new inter pretation.

Not in line with the simple picture of chemokines being responsible solely for recruiting immune cells to the injured renal tissue are findings that a) MCP-1 antibodies not only reduced cellular infiltration but also collagen expression and renal fibrosis [19]

b) mesangial cells express CXCR3 and showed a proliferative response to IP-10 [20], c) mesangial cells expressed both CCR2 and MCP-1 under high glucose conditions and with TNF present [U. Janssen *et al*., manuscript in preparation] leading to an autocrine cycle of activation; d) mouse mesangial cells showed autoinduction of chemokines through specific receptors [23] e) stimulated mesangial cells express CCR1 and at the same the respective ligand RANTES, which in turn caused a chemotactic response of MC [22]. Even more surprising from the immunologists view is the most recent finding that CCR7 is expressed in the mesangial area in biopsies of human nephritis patients, and its ligand SLC just across the GBM in podocytes [HJ Grone, pers. comm.]. So far, both CCR7 and SLC have been strictly associated with draining lymphatic capillaries and lymphnode homing of activated dendritic cells and T cells [21].

Thus, two reasons may account for the relative lack of success of anti-chemokine approaches: 1) There is still a gap of knowledge of the sequential role of different chemokines in the course of different chronic renal diseases that needs to be filled especially with respect to effect of chemokines on local cells and to human nephritis; 2) In experimental intervention studies there is still room for an optimisation of pharmacological approach in terms of the application of chemokine antagonists and the effectiveness of reaching the selected target tissue.

In human nephritis urinary chemokine measurement revealed a tight correlation between urinary MCP-1/IL-8 concentrations and disease activity of proliferative forms of nephritis including Lupus GN or IgA nephropathy and rejection status of renal transplants [6,7], although standardization of urinary chemokine sampling might need further improvement (M. Daha, pers. comm.). An accompanying avenue of better defining the role of several CCs is opened by the ERCB initiative (presented at last years meeting of this Society by M. Kretzler [8], which uses quantitative PCR of renal biopsies of nephritis patients. Nevertheless, in the near future urinary CC analysis may yield easier prognostic marker as well as a rationale for therapeutic intervention studies with safe anti-chemokine compounds. Highlighting the general interest in an anti-inflammatory therapy using chemokine blocking agents it is notable that at the US patent office server (http://164.195.100.11/netahtml/search-adv.htm) more than 450 patents related to chemokine action and >150 dealing with chemokine antagonists have been filed. From the latter the most interesting substances are small antagonistic molecules (non-peptide compounds), e.g. piperazine (Schering AG) or anilide (Takeda Ltd) derivatives (both antagonizing CCR5 ligands), which soon might enter clinical trials as anti-inflammatory drugs for oral administration. Other companies currently focussing significant resources on high-throughput screening for chemokine antagonists include Smithkline Beecham, Serono and Merck.

Immune Pharmacological Strategies to Interfere with Chemokine Action

Nevertheless, given the abundant possibilities of local chemokine action and the number of different cells involved in an inflammatory situation, it remains necessary to unravel the chemokine story more thoroughly. Our own strategy during recent years sought to define the role of local renal cells in directing a defined set of immune cells into the kidney by their specific pattern of secreted chemokines [12]. Subsequently in a Th-1 dependent model of immune nephritis in SCID mice we demonstrated that the sequence of invading inflammatory cells may be initiated by antigen-specific T helper lymphocytes encountering "their" antigen in the kidney, which subsequently directs monocytes and possibly dendritic cells towards the injured renal tissue (manuscript in review process). In accordance with these data several investigations in human and experimental GN showed that both the expression of Th-1 related chemokine receptors, CCR1, CCR2 and CCR5, in the glomerular and mainly interstitial areas as well as the urinary secretion of MCP-1 is closely associated to disease activity and phases of progression [6,9].

On the basis of these findings we have set up our anti-chemokine strategy to primarily target ligands of CCRs expressed by pro-inflammatory type-1 lymphocyte and dendritic cells. Using an eucaryotic Pichia pastoris expression system, which was proven to be effective for the expression of glycosylated cystein-rich chemokines before (17,18), we are producing N-terminally deleted mutant MCP-1 (mMCP-1_{9-76}) and human herpesvirus-8 (HHV8) -derived viral MIP-II proteins readily secreted into the Pichia supernatant after methanol induction. For our research strategy the advantage of generating protein antagonists of MCP-1 compared to seemingly more easy application of small non-peptide compounds is that we have the option to sequentially mutate the bioactivity, glycosylation or the heparan sulphate binding site while preserving the specific binding site for CCR-2 [26,17,18] (Figure 1). By using the viral broad-spectrum chemokine receptor antagonist, vMIP-II, we make use of the evolutionary strategy of this virus to evade specific, most likely type1 CD4 and CD8 guided, immune defense. As shown for other viruses this "strategy" includes chemokine agonists (for the suppressor cells?), antagonists and chemokine binding proteins or decoy receptors [11].

Uncoupling chemokine receptors represents another strategy of blocking chemokine action. The C-terminal domains of inflammatory chemokine receptors such as CXCR2 or CCR5 have been shown to contain signaling determinants, which typically map to the juxtamembranous part of this domain. It has also

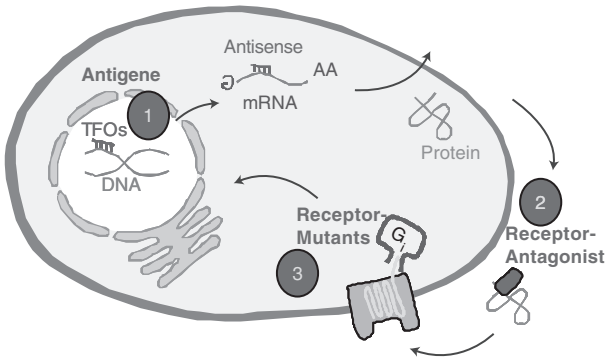

Fig. 1. Molecular targets to antagonize chemokine expression and activity. 1. "Antigene approach", i.e. nuclear targeting of triple helix-forming oligonucleotides to block CCL or CCR expression. 2. Receptor antagonists: a) mutation of bioactivity but preserving the binding site of natural ligands, b) mutation of heparan sulfate binding site to dilute tissue haptotactic gradient; c) Small, non-peptide, chemical compounds to block CCRs (see text). 3. a) Induction or transfer of decoy receptors; b) Down-regulation or uncoupling of specific CCRs; c) Blocking or interfering with cytosolic, chemokine specific signalling pathways.

been demonstrated that NFκB activation by a viral chemokine receptor homolog encoded by HHV8 was completely blocked when the final five amino acids in its C terminus were deleted [24]. Thus, the therapeutic blockade of signaling determinants at the cytoplasmic domains of chemokine receptors might be a strategy to uncouple their activities.

Moreover, inflammatory chemokine receptors have been shown to be uncoupled by anti-inflammatory cytokines, generating functional decoys [25]. This study showed that, in an inflammatory environment, IL-10 blocked the down-regulation of CCR1, CCR2, and CCR5, while uncoupling their signaling activities. Thus uncoupled receptors remain being expressed on the cell surface where they trap their pro-inflammatory ligands and thereby serve as molecular sinks and scavengers for inflammatory chemokines, removing them from the site of inflammation. Therefore, the induction of functional decoys might serve as a tool for interfering with excessive leukocyte recruitment and activation.

In addition, as depicted in Fig. 1, targeting the expression of inflammatory chemokines or their receptors on gene level is expected to be a valid strategy. The use of DNA or RNA oligonucleotides forming a triple helix with specific purine-rich duplex DNA strands has been improved considerably in recent years [13–16]. With our recent "proof of principle" study (13) we not only could demonstrate specific triplex formation with the targeted SP1 site of the MCP-1 promoter in gel shift assays vitro, but moreover achieved attenuation of RNA and secreted MCP-1 protein expression by 45%. This effect was fairly specific, because a similar SP-1 target site within the interleukin-6 promotor did not bind the TFO, and a closely related chemokine, IL-8, was not affected by the MCP-1 specific TFO [13]. Given the rather crude technique we used for applying the TFO further improvements of cellular delivery and nuclear targeting may increase the potential of these gene therapeutic anti-inflammatory approach considerably [15–16].

Perspectives

Given the far reading beneficial potential of regulating the inflammatory cell traffic in renal disease, anti-chemokine therapy might be worth considering in nephritis. Commercial interests will most likely accelerate the introduction of such compounds. However, with this contribution we also like to emphasize that there is still a gap of knowledge to be closed, which should be attempted in cooperation of basic and clinical sciences for the benefit of nephritis patients.

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Disturbed Chromatin Disposal and lupus Nephritis

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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease that is T-cell-dependent and autoantigen-driven. The nucleosome appears to be the major autoantigen, since about 50% of the pathogenic T helper cells are nucleosome specific [1], and anti-nucleosome autoantibodies have been detected in about 80% of lupus mice and SLE patients [2]. The formation of these nucleosome-specific antibodies precedes the formation of anti-DNA and anti-histone antibodies in lupus. Nucleosomes are the basic structure of chromatin and consist out of pairs of the 4 core histones H2A, H2B, H3 and H4 around which 2 strands of dsDNA are wrapped with histone H1 at the outside. Nucleosomes are formed during apoptosis by cleavage of chromatin. Together with other autoantigens nucleosomes appear in apoptotic blebs at the surface of apoptotic cells. During apoptosis autoantigens can be altered. Defective removal of apoptotic cells may lead to the increased presentation of modified apoptotic debris to the immune system. Finally, nucleosomes have an important role in the binding of autoantibodies to the basement membranes, in particular the glomerular basement membrane (GBM) [3]. In the subsequent paragraphs we will briefly review the current state of knowledge on the different processes mentioned above.

Lupus and Apoptosis

Since nucleosomes are formed during apoptosis, it is conceivable that apoptosis is dysregulated in SLE. Mouse models for SLE such as the MRL/lpr [4] and gld mice lack one of the activators of apoptosis, either the Fas receptor or the Fas ligand. However, in SLE patients, the expression of the Fas receptor and its ligand is normal, but the expression of the apoptosis inhibitors soluble Fas and Bcl-2 is increased. An increased in vitro apoptosis of lymphocytes has been shown, but this is probably due to an increased number of circulating activated lymphocytes. Despite these seemingly contrasting findings, it is clear that changes in the regulation of apoptosis can lead to apoptosis at the wrong moment and/or at the wrong place. The result of an imbalanced apoptosis may lead to the persistence of autoreactive T- and B-cells, but also to an increase in the quantity and a change in the composition of nucleosomes.

Disposal of Apoptotic Material

An increased amount of apoptotic cells or debris may also be the result of disturbances in the clearing machinery. Normally, the removal of apoptotic cells is a fast and efficient process that involves the engulfment by phagocytotic cells. An impaired clearance of apoptotic cells may lead to an increased release of apoptotic material and the subsequent stimulation of autoreactive T cells. The increased numbers of early apoptotic cells, and levels of nucleosomes and nucleosomal DNA in SLE patients confirm this. Macrophages of SLE patients have indeed a decreased ability to remove apoptotic cells [5] , but we could not demonstrate a constitutive clearance defect in premorbid SLE mice [6]. Recently, high-impact reports showed the importance of an efficient clearance of apoptotic material for the development of SLE. C1q is one of the mediators involved in apoptotic cell removal [7]. It has been shown that C1q deficient mice develop anti-nuclear autoantibodies and glomerulonephritis with an increased number of apoptotic bodies in the glomeruli [8]. Almost all patients with a C1q deficiency also develop SLE. Serum amyloid P (SAP) is another factor related to the removal of apoptotic material. SAP binds *in vivo* to apoptotic cells, apoptotic blebs and chromatin, thereby preventing autoimmunity against chromatin [9]. SAP-deficient mice show an anti-chromatin response, severe glomerulonephritis, and an enhanced autoantibody response to immunisation with chromatin [10]. SLE patients have a normal circulating level of SAP, but CRP, functionally related to SAP, is decreased during flares of SLE. The major endonuclease present at sites of high cell turnover is Dnase1, implicating a role in the digestion of apoptotic cell-derived chromatin. Mice deficient for Dnase1 indeed show an anti-nuclear antibody response and develop glomerulonephritis [11]. In patients with SLE Dnase1 activity is decreased. Especially, if phagocytosis is impaired, apoptotic cell material can be processed by antigen-presenting cells (APC), such as dendritic cells (DC). In an inflammatory microenvironment apoptotic cell derived autoantigens like chromatin are processed by APC and presented to autoreactive T cells in conjunction with co-stimulatory molecules. In conclusion, it appears that inadequate disposal of apoptotic cells may enhance or even induce the development of SLE.

Modification of Autoantigens

Structural changes of nucleosomes can make them more immunogenic. Several types of modifications associated with apoptosis and/or chromatin condensation have been described sofar. These include: i) phosphorylation [12], dephosphorylation or methylation of specific sites on the N-terminus of the core histones; ii) hyperacetylation or deacetylation of histones [13,14]; iii) ubiquitination sofar described for topoisomerase II and histone H2A [15]; iv) citrullination, the selective deamination of arginine to citrullin [16]; v) transglutaminase facilitated crosslinking of proteins like histone H2B [17]. The relevance of these changes has been exemplified by the demonstration of autoantibodies to phosphorylated autoantigens [12,18], ubiquitinated H2A [15], and anti-citrullin antibodies in rheumatoid arthritis [16]. In MRL/lpr mice a disruption in transglutamination has been described [17].

Cleavage of autoantigens by proteases during apoptosis is another mechanism of creating new epitopes [19]. Recently, another pathway of apoptosis induction has been linked to SLE. Cytotoxic T-cell-mediated apoptosis normally kills virally infected cells and tumor cells by releasing granzyme B, which in addition to other activated procaspases cleaves different host cell proteins. Granzyme B cleaves autoantigens, targeted in systemic autoimmune diseases, in unique fragments recognized by autoantibodies derived from lupus mice or patients, while non-autoantigens were either not cleaved or cleaved in a manner identical to other proteases [20]. Granzyme A is another specific protease that is induced by granzyme B and that makes the chromatin more accessible to endonucleases by complete degradation of histone 1 and cleavage of the core histones [21].

In addition to protein modifications, DNA can become more immunogenic. Reports have shown that abnormal DNA methylation and CG contents may result from apoptosis [22] and cause

increased immunogenicity. Finally, exposure to reactive oxygen species (ROS) or UV-light may induce DNA-modifications with a higher antigenicity [23]. In conclusion, autoantigens modification during apoptosis may render them more immunogenic, if they are not removed properly and are presented to the immune system.

Immunogenicity

Antigen presentation of apoptotic cell material by both macrophages and dendritic cells has been shown by the detection of nucleosome-specific T helper cells in lupus-prone mice and in SLE patients. T helper clones are specific for certain histone epitopes [24], but they assist in the production of antibodies to other nuclear antigens such as DNA (a process known as epitope spreading). Suprisingly, similar histone-specific Th clones were isolated from healthy donors. Other *in vitro* and *in vivo* experiments suggest that for the generation of Th cells and autoantibodies the amount of apoptotic cells is critical. Certain conditions enhance the breakdown of self-tolerance for nucleosomes, for example, viral infections. Studies with the polyomavirus BK show that the viral T antigen can bind to mammalian DNA and makes it more immunogenic in mice. Moreover, when virally infected cells become apoptotic, viral antigens become clustered in the same apoptotic blebs as nucleosomal antigens. This co-localisation facilitates the binding of the T antigen to nucleosomes leading to a greater immunogenicity for T-antigen-specific T cells [25]. So, apart from bypassing self-tolerance by autoantigen modification, immunogenic presentation of nucleosomal antigens to T helper cells can lead to the loss of self-tolerance for these antigens.

Deposition

The glomerular localization of autoantibodies first was explained by deposition of DNA/anti-DNA complexes. Thereafter, several more mechanistic hypotheses have been postulated to explain this deposition of autoantibodies in the glomerulus. Firstly, deposition has been explained by the cross-reactivity of mainly DNA-specific autoantibodies with glomerular components, such as heparan sulphate [26]. However, we proved that this HS-binding is mediated by nucleosomes coupled to anti-nuclear antibodies [3]. This binding of the anti-nucleosome/nucleosome complex to the GBM is mediated by the binding of the positively charged histone tails of the nucleosome to the negatively charged HS [3]. Similar binding mechanisms occur in vivo since nucleosomes and anti-nucleosome antibodies are present in glomerular deposits in human and murine lupus nephritis [27,28].

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Growth Factors and Renal Damage

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An ever increasing list of growth factors is identified that in some way contributes to renal disease. At first glance they represent an extremely complex, if not chaotic, ensemble and not only clinicians are confused by ever more names and a wealth of data on their biological activities. Given the redundancy of many growth factor actions and the myriad of their mutual interactions, it has come as somewhat of a surprise that specific antagonism of single cytokines is not only possible but also highly effective in clinical situations, the best example so far being the impressive effect of TNF-α blockade in rheumatoid arthritis or inflammatory bowel disease.

Some time ago, it has been proposed to apply the following principles before assigning a biological activity to a given growth factor [1]. Evidence should be presented that a) the factor exhibits the biological activity in cultured renal cells in vitro, b) the factor is present, overproduced and/or released in parallel with the biological effect in vivo, c) exogenous administration or endogenous overexpression of the factor induces or aggravates the biological effect in vivo, and d) antagonism of the factor reduces the biological effect in vivo. At present very few factors fulfill the above criteria and as such represent attractive candidates for therapeutic interventions in patients. This review will be limited to some growth factors, for which knowledge has advanced to a point, where first clinical studies appear within reach. The factors to be discussed include platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF).

PDGF: In the glomerulus PDGF B-chain, either as part of PDGF-BB or PDGF-AB, is the most active molecule (so far nothing is known about glomerular actions of the novel PDGF-C and PDGF-D). PDGF-B is produced within the glomerulus, released by infiltrating cells, overexpressed in many glomerular diseases and *in vitro* potently increases proliferation and matrix synthesis in mesangial cells [2]. *In vivo*, administration of PDGF-BB or the glomerular transfection with a PDGF-B cDNA results in mesangioproliferative changes without other renal pathology. Mice genetically deficient of PDGF-B or its receptor quickly die of renal abnormalities. The notable abnormality is in the glomeruli, which completely lack a mesangium [2]. Using a potent PDGF antagonist, a specific DNA-aptamer, we demonstrated that it was highly effective in reducing mesangial proliferation and matrix accumulation in experimental mesangioproliferative nephritis [3] and more recently, that this effect did not involve downstream inhibition of the TGF-β system [4]. The latter would be a potential concern, since the long term consequences of TGF-β antagonism, a growth factor with not only pro-fibrotic but also potent immunosuppressive activity, are unknown. More importantly, a few days of treatment with the PDGF-antagonist during the mesangioproliferative phase of a chronic anti-Thy 1.1 model completely prevented the subsequent development of renal failure and glomerular as well as tubulointerstitial scaring [5]. This study thereby provided the first evidence for the long held believe that specific reduction of mesangial cell proliferation *in vivo* is indeed a meaningful therapeutic approach to mesangioproliferative disease. Importantly, both experimental renal studies [6] as well as phase I and II studies with orally available PDGF-B antagonists in tumor patients have shown little toxicity [7].

VEGF is a homodimeric protein consisting of at least five different molecular species, having 121, 145, 165, 189, and 206 amino acids respectively. In normal kidney, VEGF expression, mainly of the 165-isoform, is confined to podocytes, distal duct epithelia, and collecting-duct epithelia. It is also inducible in mesangial cells, where it is overexpressed during mesangioproliferative glomerulonephritis. VEGF is a potent mitogen, chemotaxin and survival factor for endothelial cells. These activities constitute an essential part of VEGF's angiogenic activity [8]. In experimental glomerulonephritis as well as in a variety of human glomerular diseases capillary repair with features of angiogenesis occurs and $VEGF_{165}$ has been shown to be an essential component of this process [9]. Vice versa, administration of VEGF has recently been demonstrated to augment glomerular endothelial regeneration [10], rendering VEGF an attractive candidate for the treatment of e.g. thrombotic microangiopathies.

HGF, also referred to as scatter factor, is a multifunctional cytokine with mitogenic, anti-apoptotic, morphogenic and motogenic actions. It can induce tubulogenesis and as such is important in the development of the kidney or renal cyst formation [11]. In addition to acting locally, HGF is also a circulating (i.e., endocrine) molecule. HGF is produced by fibroblasts, microvascular endothelial cells, and mesangial cells. Upregulation of HGF synthesis and/or its receptor c-met in the kidney occurred in various situations, such as uninephrectomy, acute toxic renal injury, renal transplant rejection, renal ischemia and diabetic renal hypertrophy. Systemic administration of HGF stimulated renal tubular regeneration, accelerated recovery of renal function, and reduced mortality in experimental acute renal failure [12,13]. In experimental mesangioproliferative glomerulonephritis, HGF, like VEGF, enhanced the angiogenic capillary repair. In a spontaneous mouse model of renal failure as well as in obstructive nephropathy treatment with recombinant HGF almost completely prevented the onset of tubulointerstitial fibrosis and attenuated the progression of glomerulosclerosis [14,15], possibly via counteracting the pro-fibrotic effects of TGF-β. However, before proposing HGF administration for the therapy of acute or chronic renal failure, one needs to bear in mind that transgenic mice, which markedly overexpress HGF under the metallothionin promoter, paradoxically develop glomerulosclerosis and tubular cystic disease as well as tumors of various origins [16].

The above observations clearly demonstrate that out of the apparent chaotic ensemble of growth factors in renal disease, it is possible to extract individual dominant functions of some molecules, which can either be antagonized or augmented as part of new, specific approaches to renal disease.

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Rheopheresis for Age-Related Macular Degeneration

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Key Words

Therapeutic apheresis, Rheopheresis, age related macular degeneration, dry AMD, drusen

Introduction

Age-related macular degeneration (AMD) is the leading cause of severe visual loss and legal blindness in people older than 65 years in the Western world. There are two types of macular degeneration: the nonexudative form (dry AMD) and the exudative form (wet AMD). Clinical manifestations appear as drusen, atrophy of the retinal pigment epithelium (RPE) and choriocapillaris, i.e. dry AMD, RPE detachment, and choroidal neovascularization (CNV), i.e. wet AMD. 10–20% of patients with dry AMD progress to the wet form, with number, size and confluence of drusen being risk factors for that development [1]. Patients with wet AMD account for app. 25% of total AMD patients [2]. In Germany in the year 2000 the prevalence of patients between 43 and 86 years with findings of wet AMD was app. 430.000, the equivalent prevalence of legally blind AMD patients was app. 20.000, accounting for 15–32% of all cases of blindness [3].

Pathogenic Mechanisms of AMD

The pathogenesis of AMD is not yet fully understood, but a hypothetical sequence of pathogenic events is consistent with known data. Models for AMD pathogenesis include RPE and Bruch's membrane senescence, genetic defects, oxidative insults, and ocular perfusion abnormalities [2]. In epidemiological studies cholesterol, fibrinogen, and α2-macroglobulin have been established as risk factors for AMD [4,5,6]. In a cross sectional study patients with dry and wet AMD were compared to healthy controls investigating markers of angiogenesis, hemorheology, and endothelial dysfunction. Levels of vascular endothelial growth factor (VEGF), plasma viscosity, fibrinogen, and von-Willebrand factor (vWF) were measured in combination with a variety of standard laboratory parameters [7]. Median plasma VEGF ($p = 0.019$), mean vWF ($p < 0.001$), mean fibrinogen ($p < 0.001$), and mean plasma viscosity ($p < 0.001$) were elevated in AMD patients.

In the vascular model for AMD pathogenesis proposed by Friedman, it is hypothesized that lipid deposition in sclera and Bruch's membrane leads to scleral stiffening and impaired choroidal perfusion, which would in turn adversely affect metabolic transport function of the RPE [8]. The impaired RPE cannot metabolize and transport material shed from the photoreceptors, leading to accumulation of metabolic debris and drusen. The model is in accordance with a huge body of findings demonstrated by fluorescein and indocyanine green angiographic methods, laser Doppler flowmetry, and color Doppler imaging [2]. Irrespective whether the microcirculatory derangements found in AMD are primary or secondary, this model represents a consistent framework for the development of both the nonexudative and exudative forms of AMD [2].

Cellular functions of RPE is depending upon oxygen concentration [9]. Phagocytosis is a major function of RPE cells and is essential to maintain homeostasis of the microenvironment in the eye. Drusen form within Bruch's membrane, a stratified extracellular matrix situated between the RPE and choriocapillaris. It has been concluded, that the senescent RPE accumulates metabolic debris as remnants of incomplete degradation from phagocytosed rod and cone membranes and that progressive engorgement of these RPE cells leads to drusen formation with subsequent progressive dysfunction of the remaining RPE. Blood flow can have a direct functional relationship with tissue cells via shear stress. In terms of RPE function, decreased blood flow could result in decreased RPE phagocytosis by insufficient tissue oxygenation, and reduced induction of TGF-β in the vessel

wall via reduced shear stress [9,10,11]. Bruch's membrane thickened with drusen then could facilitate the development of CNV (2). Whatever the initial stimulus for CNV formation might be, it is clear that angiogenic growth factors are ultimately involved. The macular region including the fovea is an avascular zone, much thinner than the rest of the retina, and receives nourishment by diffusion from the surrounding vasculature and choriocapillaris. Angiogenic and antiangiogenic factors in the retina coordinate vascular flow and regeneration with the corresponding metabolic requirements of the retina. Most important are vascular endothelial growth factor (VEGF) and pigment epithelium derived factor (PEDF), both regulated by tissue oxygenation [12]. Expression of VEGF is induced by hypoxia, thus promoting neovascularization, PEDF is induced by increase of oxygen, thus inhibiting neovascularization. The integrity of the vessel wall under quiescent conditions as well as its appropriate responsiveness under conditions of stimulation, inflammation or vascular injury is controlled by a number of adhesive interactions. Two major adhesive proteins relevant for haemostatic mechanisms co-localized in the ECM of the vessel wall have to be mentioned: von Willebrand factor and vitronectin [13].

By immunohistochemistry and RT-PCR analysis it was shown that vitronectin is a major constituent of human drusen, and that it is expressed by local RPE cells [14]. More common extracellular matrix (ECM) components such as laminin, fibronectin, collagens, and proteoglycans were not detected, indicating, that drusen-associated vitronectin is the result of selective accumulation. Vitronectin is present in high concentrations in plasma and is also common in ECM. Functionally it is related to processes of thrombosis, fibrinolysis, inflammation, and cellular adhesion. Self-association of vitronectin results in the formation of multimeric species of the protein [15]. The balance between monomeric and multimeric forms of vitronectin is important for pathophysiologically relevant changes of ECM sites and fibrinolytic state in plasma [16]. The binding and deposition of vitronectin to Bruch's membrane could compromise the exchange of metabolites between the choriocapillaris and the RPE, eventually leading to RPE and photoreceptor cell dysfunction and degeneration. Alternatively it is conceivable that drusen form as a consequence of RPE dysfunction and deterioration. RPE cells or byproducts of abnormal RPE and/or photoreceptor cell metabolism could serve as nucleation sites for the deposition for proteins such as vitronectin. In a mouse modela vitronectin receptor antagonist could reduce neovascularization in dose-dependent fashion, indicating that vitronectin deposition might be finally a contributing factor of neovascularization [17].

Therapeutic Options for AMD

Currently only the wet form of AMD is generally regarded as treatable by available therapeutic methods [2]. In the majority of AMD patients the therapeutic situation is very unsatisfactory, especially for patients with dry AMD. Even not all patients with wet AMD are eligible for the following treatment options: laser therapy including standard laser photocoagulation for extrafoveal classic CNV, transpupillary thermotherapy (TTT) for occult subretinal/subfoveal CNV, and photodynamic therapy (PDT) for subfoveal or predominantly classic subretinal CNV, external beam irradiation for subfoveal and occult CNV, and surgical procedures like removal of neovascular membranes or macular rotation. Laser photocoagulation and TTT are currently tested for dry AMD. However, reports on the occurrence of increased neovascular lesions after laser photocoagulation or TTT could indicate that both approaches might turn out to be very unfavourable in the subsequent course of AMD [18,19, 20,21]. From a pathophysiological point of view, regarding angiogenic growth factors both treatment modalities seem to induce VEGF rather than PEDF. In conclusion from the current situation, successful therapy for more subgroups of patients with AMD is urgently needed.

Rheopheresis

Rheopheresis is a safe and effective application of membrane differential filtration (MDF) for extracorporeal hemorheotherapy [22]. The elimination of an exactly defined spectrum of high-molecular weight proteins results in the reduction of blood and plasma viscosity as well as erythrocyte and thrombocyte aggregation and flexibility. Pulses of lowering blood and plasma viscosity leads to improved blood flow, subsequently inducing sustained improvement of microcirculation, which means recovery of organ and tissue function. In this context microcirculation has to be considered not only as patency of capillary blood vessels, but in terms of the complete interactive network between plasma, blood cells, cells of the vessel wall, e.g. endothelial cells, vascular smooth muscle cells, and fibroblasts, and the compartments of the surrounding tissue, e.g. cells and extracellular matrix.

Rheopheresis for AMD – Clinical Trials

Data from two prospective, controlled, and randomized clinical trials are available for the treatment of AMD patients with Rheopheresis [23,24,25]. In the study of the University of Cologne 40 patients were included. 20 patients received 10 Rheopheresis treatments over a period of 21 weeks. Comparing initial and final visual acuity in Rheopheresis and control patients a mean difference of 1.6 EDTRS lines was detected after the treatment series, which was a statistically significant difference with $p < 0.01$ [23]. Patients with soft drusen and no CNV had the best therapy results. Electroretinogram analysis showed significant improvement of photopic a-wave and the flicker ERG, equivalent to functional improvement of the central photoreceptor complex. 30 patients with dry AMD and soft drusen were included in a three-armed, sham-controlled, randomized clinical trial conducted at the University of Utah, Salt Lake City. With 10 Rheopheresis treatments 40% of patients showed improvement in at least 3 out of the following 4 parameters: $\geqslant$ 2.5 ETDRS lines best spectacle corrected visual acuity (BSCVA) in the study eye, $\geqslant$ 2.5 ETDRS lines in both eyes, 20% improvement of reading ability in the Pepper visual skills for reading test, and 20% improvement of quality of life tested by the VF14 [24,25]. In a case series including 10 patients with early AMD improvement of visual acuity essentially identical to the Cologne trial could be confirmed [26]. From 11 patients results of long-term treatment were reported, demonstrating that the therapeutic effect of the initial treatment series can be maintained over more than 2 years [27]. Eyes suffering from dry AMD had a mean improvement of visual acuity of 2.5 EDTRS lines after 24 months. Approximately 12 months after the initial treatment series 2–4 booster treatments could be considered depending upon the individual course [27]. In summary these data can be graded as *class I* with respect to the categories of evidence-based-medicine.

Rheopheresis – Safety Issues

All methods of extracorporeal blood purification can be associated with adverse reactions, which all are well known due to the

huge worldwide experience with hemodialysis, plasma exchange, immunoadsorption, and LDL-apheresis. Hypotension, allergic reactions due to blood membrane interactions, hemolysis, or events associated with anticoagulation have to be mentioned. Safety of MDF was analyzed including data from 1702 ambulatory MDF-LDL-apheresis treatments of 52 patients [28]. In 98% of MDF-treatments no adverse reactions occurred. In 2% hypotensive episodes were observed, no severe adverse events occurred [28]. In a trial of Rheopheresis in 10 patients with ischemic stroke also no severe adverse events were reported [29]. In the controlled trial of the University of Cologne 20 patients with a mean age of 72 years received in total 100 Rheopheresis treatments [23]. Hypotension was observed in 6%, hemolysis in 2.5% of treatments. A current RheoNet-registry analysis in June 2001 including 1,009 Rheopheresis treatments in 219 patients with the mean age of 69,8 years showed adverse reactions in 1.5% of treatments, including mainly hypotensive episodes, and only few allergic reactions or observations of hemolysis. No symptomatic hemolysis occurred (30 and R. Klingel, unpublished data). For comparison in chronic hemodialysis symptomatic hypotension is reported in upto 20% of treatments [31]. Based on interdisciplinary cooperation between ophthalmology and nephrology Rheopheresis can be regarded as a very safe treatment, even for elderly patients with AMD.

Rheopheresis in AMD – *"How Does it Work?"*

Extracorporeal plasma therapy was not yet used in ophthalmology and therefore requires not only discussion with respect to methodology, but also regarding the mechanism to explain the therapeutic benefit. The knowledge of AMD pathogenesis can be summarized, that AMD at cellular and molecular levels is at least in part a microcirculatory disorder of the retina. Therefore it seems to be reasonable to use Rheopheresis, which can successfully treat diseases with impaired microcirculation [22]. It is important for the understanding of the therapeutic potential of Rheopheresis, that the single pulses of plasma protein elimination with associated reduction of plasma viscosity can result in sustained improvements of microcirculation. This of course is a hypothesis, but it is confirmed by available clinical data for AMD, ischemic diabetic foot syndrome, and sudden deafness.
Rheopheresis directly targets risk factors and pathophysiologically relevant factors of AMD by lowering plasma viscosity, and eliminating fibrinogen, cholesterol, von-Willebrand factor, α2-macroglobulin, and probably multimeric vitronectin. RPE phagocytic function is regulated by tissue oxygen concentration [9]. Rheopheresis treatment results in sustained improvement of tissue oxygenation induced by the repeated therapy pulses, as recently confirmed in a pilot trial in patients with ischemic diabetic foot syndrome [32]. In AMD Rheopheresis could improve RPE phagocytic function directly by the increase of tissue oxygenation, and additionally via shear stress mediated induction of TGF-β [9, 10, 11]. Rheopheresis could also correct the imbalance of the growth factor opponents VEGF and PEDF in favor of inhibition of angiogenesis. This could result in the prevention of AMD progression. From a pathophysiological point of view this could be an advantage over laser-based treatments like photocoagulation or TTT, which in principle are destructive, and therefore might worsen the VEGF/PEDF imbalance. The hypothesis that Rheopheresis might re-balance the angiogenic growth factor sytems of VEGDF and PDF is clinically supported by the finding, that also diabetic retinopathy improved from Rheopheresis treatment [33].

Indication for Rheopheresis in AMD

In total more than 300 patients with AMD have been treated by extracorporeal hemotherapy and confirmed the study results in clinical practice (Brunner *et al*.: personal communication). At present Rheopheresis should be regarded as complementary therapeutic option. If the patient has an indication for laser photocoagulation, photodynamic therapy, or surgery, this should be considered prior to Rheopheresis. The current recommendation for the indication of Rheopheresis in AMD is part of practice guidelines which are continuously updated by data from clinical trials and analysis of the RheoNet-registry [30]. The eye selected for Rheopheresis treatment should have the following characteristics: 1. better eye of the patient with BSCVA $\leqslant$ 0.7, 2. dry AMD with multiple soft drusen, and 3. no subfoveal neovascularization. Two schedules for 8-10 Rheopheresis treatments can be chosen according to individual patient needs, which seem to be equally effective based on available experience. Schedule 1 consists of two Rheopheresis treatments per week, followed by 4-week intervals, schedule 2 consists of two Rheopheresis treatments in the first week, followed by single treatments every two weeks.

Conclusion

In the majority of AMD patients the therapeutic situation is very unsatisfactory, especially for patients with dry AMD. Rheopheresis is a safe and effective therapeutic modality of extracorporeal hemorheotherapy to treat microcirculatory disorders, and represents a novel therapeutic approach for patients with dry AMD. A series of Rheopheresis treatments with effective pulses of decreased plasma viscosity and elimination of microcirculatory relevant high-molecular weight proteins can induce sustained improvement of the progressive clinical course of dry AMD. The implementation of Rheopheresis into clinical practice is following the needs of evidence-based medicine. The RheoNet-registry and the development and continuous update of therapy guidelines provide a framework of appropriate quality management to this interdisciplinary therapy concept. The hypothesis how Rheopheresis can be effectively used in AMD patients is closely associated with our current knowledge of pathogenic mechanisms of the developmemt and progression of AMD.

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Indications for Therapeutic Apheresis in Neurologic Diseases

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Key Words

Therapeutic apheresis, plasma exchange, immunoadsorption, membrane differential filtration, Refsum's disease, Myasthenia gravis, Guillain-Barré-syndrome, Multiple sclerosis

Introduction

Methods of therapeutic apheresis are effective to remove pathogenic agents from the bloodstream. Progress of our understanding of underlying pathogenic mechanisms led to the empirical implementation of therapeutic apheresis for a number of neurologic diseases, which since has been confirmed in many fields by a substantial number of controlled clinical trials to meet the criteria of evidence-based-medicine. Regarding methodology standard plasma exchange is becoming at least in part replaced by selective immunoadsorption, avoiding the need for substitution of plasma products with the associated low but not negligible risk of virus transmission [1]. In neuroimmunological diseases caused by autoimmune processes, especially involving autoantibodies immunoadsorption competes with the intravenous administration of immunoglobulins (IVIG). The mechanisms of the beneficial action of high doses of normal IgG in antibody-mediated disorders are far from being fully understood [2]. However, all these proposed molecular and cellular mechanisms, such as the upregulation of the cellular transport receptor for IgG, named FcRn, will have to be studied further until their effect can become clinically relevant. Therefore, in general extracorporeal methods might be preferred, if immediate response is required. The effect of immunoadsorption is not solely related to direct removal of pathogenic agents, an immunomodulatory effect must also be considered [3]. The neuroimmunological diseases in which therapeutic apheresis has gained most interest are acute idiopathic demyelinating polyneuritis, known as Guillain-Barré-syndrome (GBS), Myasthenia gravis (MG), and Multiple sclerosis (MS). Miller-Fisher-syndrome associated with anti-Ganglioside antibodies, Stiff-man-syndrome associated with

glutamate-decarboxylase antibodies, and paraneoplastic Lambert-Eaton-syndrome associated with antibodies against pre-synaptic calcium channels could be additionally mentioned. Refsum's disease is a metabolic disorder with major neurological symptoms, in which the extracorporeal elimination of high-molecular-weight plasma proteins represents an effective therapeutic approach. In intensive care the vascular access for acute therapeutic apheresis will be mostly a central venous catheter, peripheral veins are the appropriate vascular access sites for long-term ambulatory treatment.

Myasthenia Gravis

In Myasthenia gravis, autoantibodies block the postsynaptic acetylcholine receptors of muscle as the major pathogenic mechanism. Whereas mild and moderately severe cases of MG can successfully be treated by thymectomy, pyridostigmine, corticosteroids, and azathioprine, these treatments are not sufficient in severe cases. Since 1975, plasma exchange treatment for severe MG has been in use, and became accepted especially as treatment for patients with problems not adequately controlled by standard therapy. A clinical trial including 60 patients, demonstrated in 1983 that the majority of patients previously refractory to conventional and immunosuppressive therapy responded well to plasma exchange, and that their clinical improvement lasted for about 2 months after completion of plasma exchange [4]. Results of two clinical trials using a tryptophan-linked polyvinyl alcohol gel immunoadsorber showed that selective immunoadsorption seemed to be equally effective as plasma exchange [5,6]. Refractory patients with severe generalized MG improved rapidly after 4–5 treatments, and the effect was sustained over a period of at least 6 weeks. In a randomized trial with MG patients suffering from acute exacerbations of MG, plasma exchange and IVIG were equally effective [7].

Concluding from these study results therapeutic apheresis performed as plasma exchange or selective immunoadsorption is indicated in cases of severe MG or myasthenic crisis with rapidly progressive weakness, especially when respiration is impaired [5,8,9]. In these patients additional esophageal paresis can lead to severe pulmonary complications by aspiration. Plasma exchange and selective immunoadsorption can be regarded as equivalent in their efficacy to treat patients with severe MG, as recently shown in a controlled clinical trial with 19 patients [10]. After 3–5 treatments acute MG crisis was controlled with both methods. The advantage of extracorporeal elimination is the rapid therapeutic effect compared to IVIG. Immunoadsorption is not only a very effective treatment option for acute MG, but can also have a place in the chronic rehabilitation of selected MG patients, if combined drug therapy is either not sufficiently effective or not well tolerated. The case report of a 51-year old patient suffering from severe MG (Ossermann IV) since 9 years illustrated this experience [11]. After surgery of thymoma and radiation, the patient was treated with pyridostigmine and several immunosuppressive drugs cortisone, azathioprine, cyclosporine A, methotrexate, or cyclophosphamide without improvement of the clinical course. Also a therapy trial with plasma exchange was not tolerated. Since 5 years then the patient received drug treatment combined with weekly immunoadsorption using a gel column based on tryptophan-linked polyvinylalcohol resin resulting in a significant reduction of autoantibody and a significant improvement of patient's morbidity with respect to hospitalization and quality of life.

Guillain-Barré-syndrome

The Guillain-Barré-syndrome (GBS) is a clinically defined disease entity for which a set of diagnostic criteria was established that includes clinical, electrophysiological, and cerebral spinal fluid findings. The hallmark of the syndrome is a relatively symmetrical polyneuropathy of acute onset that predominantly affects motor fibres of the peripheral nervous system. The acute monophasic disease reaches its nadir in less than 4 weeks followed by slow recovery over many months to 1 year. A large number of immunological features were described, and it is now accepted that the disease is caused by an autoimmune process in which activated T-lymphocytes play an important role [12]. The pathogenic mechanisms have not been established completely, but in many cases an antecedent infection by campylobacter jejuni leads to the production of antibodies directed against certain epitopes of the bacterium that also destroy the myelin sheath of the peripheral nerve [13]. There is ample evidence that GBS is merely a clinical phenotype that actually can be caused by a number of different pathogenic mechanisms. This might explain the various subtypes of GBS such as axonal GBS or pure motor GBS. The observation that the spectrum of ganglioside autoantibodies varies with the clinical picture of GBS confirms the pathogenic relevance of these autoantibodies [13].

Approximately 10% of patients require intensive care treatment including respirator treatment due to the paresis of respiratory muscles. Prognosis in general is favorable although up to 10% of the patients suffer from permanent impairment, usually in the form of distal atrophic muscle paresis and contractures. The treatment of GBS is directed toward 2 main goals: (1) life support with intensive care treatment followed by optimal physical therapy, and (2) the attempt to stop the autoimmune process destructing the myelin sheaths of peripheral nerves. Aggressive supportive care alone led to a dramatic reduction of mortality, which has dropped from about 50% to less than 10% in patients requiring ICU treatment. Immunomodulatory treatment methods shorten the course of the disease but do not influence mortality markedly. Two large controlled multicenter studies demonstrated a significant acceleration of improvement of muscle function after extracorporeal plasma exchange when compared to patients with supportive treatment only [14,15]. The number of plasma exchange treatments should be adapted to disease severity [16]. Available data indicate that in no case more than 4 plasma exchange treatments seemed to be of therapeutic value [9]. Subsequently, immunoadsorption has been also implemented in the treatment of GBS. Equal efficacy of immunoadsorption compared to plasma exchange was shown in a recent study investigating 45 patients with GBS [17]. In 2 controlled studies equal efficacy of plasma exchange and IVIG was established [18,19]. A potential benefit of combined immunoadsorption with IVIG remains controversial [9,17,20]. Therefore in general preference is currently given to IVIG. In situations when respiratory failure might develop it is an important advantage to have the therapeutic effect more rapidly, and extracorporeal therapy should be preferred in such an early disease state [21]. Due to the monophasic course of the disease extracorporeal therapy after 2–4 weeks can no longer result in a beneficial course of the patient.

Chronic Inflammatory Demyelinating Polyradiculoneuropathy (CIDP)

CIDP is an immune-mediated neuropathy that affects the peripheral motor and sensory nerves. Chronic progressive forms can be

distinguished from chronic relapsing forms. The clinical picture is made up of symmetrical motor weakness, sensory involvement, and hypo- or areflexia. Clinically and regarding its pathogenesis CIDP resembles to a certain extent acute GBS. Steroids, immunosuppressive drugs, IVIG and plasma exchange have been shown to be effective therapeutic options [22,23]. Data from controlled clinical trials are available for steroids, plasma exchange, and IVIG. Efficacy of azathioprine, cyclophosphamide, and immunoadsorption is based on case series [22,23, 24,25]. Therapy must be optimized to individual needs. Due to the chronic course of the disease immunosuppression can be associated with severe side effects. The cases of two patients, refractory to several therapy regimens, who have been treated successfully for 3 and 5 years with plasma exchange or immunoadsorption, document the individual benefit of these extracorporeal therapeutic options in long-term treatment [26].

Refsum's Disease

Phytanic acid storage disease (known as Refsum's disease) is caused by inherited defects in the metabolic pathway for phytanic acid, a dietary branched-chain fatty acid. Refsum's disease has an autosomal recessive inheritance, and is caused by mutations of the phytanoyl-CoA-hydroxylase-gene [27]. Phytanic acid accumulates in fatty tissues, including myelin sheaths, in the inner eye, in heart, liver, and kidneys resulting in impairment of organ function [28,29]. Over time, affected individuals may develop classical diagnostic features of retinitis pigmentosa, cerebellar ataxia, peripheral polyneuropathy, and elevated protein levels in cerebrospinal fluid, as well as liver, kidney and heart disease. 65% of phytanic acid is bound to lipoproteins especially LDL, VLDL, and HDL [30]. Dietary restriction of phytanic acid is useful in preventing acute attacks and arresting the progression of organ impairment. Therapeutic apheresis has been shown to be particularly useful for rapidly lowering plasma phytanic acid levels during acute attacks and may play a significant role as maintenance therapy [29]. Phytanic acid can be effectively eliminated from plasma by methods of selective LDL-apheresis, in particular with membrane differential filtration [MDF, 31,32,33]. MDF uses size-selected filtration of high-molecular-weight plasma components after membrane plasma separation and represents a safe and well established method of therapeutic apheresis [34]. The term double-filtration-plasmapheresis (DFPP) is used synonymously. Mean reduction of 50% plasma phytanic acid can be achieved per single treatment session [31,32,33]. Successful maintenance treatment for 2–4, 5 years was reported in 4 patients, including monozygotic twins [33]. MDF treatment every 1–3 weeks resulted in excellent neurological rehabilitation.

Multiple Sclerosis

Multile sclerosis (MS) is a common cause of severe neurologic disability in adults of northern European origin. There is general agreement that MS is an autoimmune disorder, although recent studies have raised the possibility that there is more than one pathway to the final pathological lesions, and that the different pathways may predominate in different clinical forms of MS, e.g. relapsing-remitting, primary or secondary progressive [35]. Therapy of MS which is mostly based on steroids, had achieved significant progress after the results of several placebo-controlled clinical trial demonstrated the positive effect of interferon beta 1a and 1b. Plasma exchange has not been widely accepted as a major treatment for multiple sclerosis (MS) and other inflammatory demyelinating diseases. Several uncontrolled studies have suggested that patients with severe attacks of MS may improve rapidly after plasma exchange. A recently completed randomized, sham- controlled, crossover clinical trial investigated plasma exchange in 22 patients with idiopathic inflammatory demyelinating disease including 12 patients with MS [36,37]. 42% of patients experienced moderate or greater functionally important neurological recovery over 2 weeks of active treatment administered every other day, while only 6% of patients experienced similar improvement in the sham treatment group. 3 patients who failed the sham treatment improved rapidly after crossover to active treatment; no patient who failed active treatment improved after crossover to sham. This study illustrated the importance of uncontrolled case reports of treatment efficacy to design randomized clinical trials concerning the treatment regimen and included patient population. Plasma exchange might be considered for patients with idiopathic inflammatory demyelinating disease in situations of acute relapses of progressive forms [35,37]. Confirmation of this opinion is needed, by future clinical trials. The role of selective immunoadsorption remains to be elucidated [38].

Conclusion

As in other medical disciplines using therapeutic apheresis, extracorporeal treatment mostly is not the general treatment of first choice, but provides an excellent option to solve therapeutic problems in selected patients. Knowledge of underlying pathogenic mechanisms is the basis for effective application of a particular method of therapeutic apheresis in a specific disease. Therapeutic apheresis is a valuable therapeutic option in neurology including intensive care as well as long-term ambulatory patient care. Considering the present knowledge, plasma exchange, immunoadsorption and IVIG are therapeutic options for neuroimmunological diseases with essentially equal safety and efficacy. The decision to favor one of the treatments will be influenced by the individual needs of the patient, by the availability of the various treatment modalities, and on cost, which can vary considerably under different circumstances. Plasma exchange should be replaced by selective immunoadsorption whenever possible to minimize substitution of plasma products with the associated risk of viral contamination. Future developments of therapeutic apheresis might be directed toward a more specific adsorption or other elimination techniques, that would enable a more limited and specific removal of plasma components. For this technique, the circulating pathogenic agents would have to be characterized in more detail. Progress in our understanding of pathogenic mechanisms might also guide the application of therapeutic apheresis to new indications.

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Leukocytapheresis (Cellsorption) for inflammatory bowel disease

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Key Words

Therapeutic apheresis, leukocytapheresis, ulcerative colitis

Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic inflammatory bowel diseases (IBDs) of unknown etiology. In the last few years, multi-factorial pathogenic mechanisms have been discussed for both UC and CD. These include immunological, environmental, genetic, and microbiological factors. UC and CD are characterized by abnormal immuno-regulatory T cells. It has been suggested that contact with antigens from the normal intestinal flora leads to T-cell hyper-responsiveness [1,2].

The therapeutic modalities used to treat IBD depend on disease activity and bowel involvement and act at various sites along the

immunologic and inflammatory pathways. Although traditional medical therapies, such as sulphasalazine, aminosalicylates (5-ASA) and corticosteroids, continue to be cornerstones in the management of IBD, immunomodulators, such as azathioprine and 6-mercaptopurine are increasingly important in the setting of steroid-resistant and steroid-dependent disease. Furthermore, other immunomodulators (e.g. methotrexate, tacrolimus, monoclonal anti-TNF-antibodies) may also be beneficial in the treatment of disease.
Because only 80% of patients respond to conventional medical therapy [3], surgery is often indicated for those patients with insufficient response to these standard therapies. In addition, the long term use of corticosteroids often causes serious side effects such as osteoporosis and diabetes mellitus. Therefore, additional therapeutic alternatives are urgently needed in order to optimize the therapy for the individual patients.

Leukocytapheresis for Ulcerative Colitis

Recently, leukocytapheresis with a leukocyte removal filter (LCAP) has been applied to treat various diseases such as cancer, rheumatoid arthritis, ophthalmic Grave's disease, Behcet's disease, and pemphigus vulgaris [4]. LCAP utilizes an extracorporeal circulatory system and the selective removal of leukocytes.
Elevated circulating levels of leukocytes are seen in patients with inflammatory bowel disease. Both ulcerative colitis and Crohn's disease exhibit the classic pathological characteristics of chronic inflammation and defects in mucosal immunoregulation: that is, infiltration of the intestinal mucosa and submucosa with white blood cell infiltration, including monocytes, lymphocytes, polymorphonuclear neutrophils and plasma cells. Granulocytes are the first cells mobilized to the sites of inflammation. There they interact with lymphocytes to orchestrate the inflammatory response. The direct removal of white blood cells from the circulation by leukocytapheresis reduces the numbers available to enter the inflamed mucosa and may be a plausible therapeutic approach for IBD. In addition, the most important therapeutic goal should be the induction of tolerance towards inflammation inducing antigens. It has been suggested that by using leukocytapheresis to reduce the number of antigen sensitized lymphocytes in circulation as well as in the mucosa, it is possible to establish a level of tolerance in the mucosal immune system.
Currently, a prospective randomized clinical pilot trial is taking place in Rostock, Germany, which is investigating the safety and effectiveness of leukocytapheresis in comparison with standard steroid prescription for patients with chronic steroid-resistant or steroid-depending ulcerative colitis. The leukocytapheresis column used is the Cellsorba FX, manufactured by Asahi Medical, Tokyo, Japan, which consists of a polypropylene non-woven polyester fiber filter and which removes upto 99% of both circulating granulocytes and monocytes and about 70% of circulating lymphocytes, especially activated leukocytes [5].
The course of this study consists of an intensive therapy phase with five weekly LCAP sessions with a treated blood volume of 4000 ml. Before and during the intensive phase, patients received a low-dose steroid therapy (10 mg/day) with a constant dosage of the other concurrent medication. The efficacy is monitored using the Clinical Activity Index (CAI, Rachmilewitz), endoscopic findings, and laboratory tests including flow cytometry and cytokine measurements. Patients, who achieve remission or respond after 5 LCAP-treatments during intensive therapy are randomized into two groups. After randomization steroid dose is tapered down by 2.5 mg/week in both groups. In the therapy group, patients continue with Cellsorba FX- leukocytapheresis performed once every 4 weeks. The control group is treated with steroids/salicylates modified according to the patient's condition. Patients are currently enrolled in this trial and the results will be reported in the near future.
Positive results from several studies in Japan indicate that leukocytapheresis can not only be used for chronic steroid-resistant cases of ulcerative colitis in combination with steroid therapy [4,6,7,8], but also in patients with severe UC unresponsive to an intensive intravenous steroid regimen [9].
Just recently, a controlled, randomized multi-center trial was completed in Japan. All told, more than 100 patients with moderate to severe ulcerative colitis were randomized into two groups. The LCAP group received an intensive LCAP therapy of 5 weekly treatments followed by 11 LCAP sessions with a gradually decreasing frequency to once every 4 weeks. The control group received prednisolone treatment of 30–80 mg/day tapered down to 0–15 mg/day. Global judgement of efficacy was 74% in the LCAP group, compared to 38% in the prednisolone group . No severe side effects occurred in the LCAP group [Sawada *et al*., unpublished data].
The results of another multi-center clinical trial using a granulocyte and monocyte filter containing cellulose acetate beads (G-1 Adacolumn) were recently published. In this trial 53 patients with ulcerative colitis mainly refractory to conventional therapy received five apheresis sessions for five consecutive weeks in combination with prednisolone. At the end of the treatment phase 58.5% of the patients experienced remission or improvement and the dose of prednisolone could be reduced. No severe side effects were observed [10].
It is been speculated that LCAP therapy targets the underlying pathogenic mechanisms of the disease, particularly in the area of cell-mediated immune responses. The rapid removal of a high number of circulating leukocytes from the peripheral blood causes some kind of alteration of phenotype, particularly of the expression of leukocyte homing receptors in the bowel [7,11]. Changes in the expression of membrane adhesion molecules may reduce the ability of circulating leukocytes to migrate to the inflamed mucosa [12]. The clinical improvement and the findings on flow cytometry published in many studies suggest that LCAP exerts an immunomodulatory effect.
The potential therapeutic benefits of LCAP are the interruption of the acute disease state, maintenance of remission and steroid sparing effects. The exact position of leucocytapheresis within the complete spectrum of disease management options for ulcerative colitis has yet to be determined, but these early results definitely confirm the merit of further research into this promising therapeutic option.

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LDL-Apheresis – Do we need an update? First Steps towards the Establishment of a German LDL-Apheresis Registry

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Key Words

LDL-apheresis, indication for LDL-Apheresis, hypercholesterolemia, apheresis registry

Introduction

LDL-Apheresis is an important and safe tool for treatment of patients suffering from coronary heart disease (CHD) due to severe hyperlipidemia. For several reasons, the implementation of this therapy is becoming increasingly difficult:

1. Reimbursement of costs through health insurance companies is restrictive.
2. The limited budget of General Practitioners is already overstretched due to the numerous lipid-lowering therapies and lipid diagnostics now available.
3. Experts or central institutions with the appropriate knowledge and expertise are commonly not known or are difficult to reach.
4. The indications for LDL-apheresis treatment can only be assessed through a time-consuming process. It is, therefore, not uncommon, that a delay of more than one year occurs from the diagnosis and initial indication for LDL-apheresis to the actual initiation of extracorporeal treatment.

In addition to a "political" discussion on the indications for LDL-Apheresis treatment that takes place in some lipid-advisory committees, there is ongoing discussion into the costs for the reimbursement of this extracorporeal treatment. New results from lipid-lowering studies have generally not been taken into account.

The German guidelines ("NUB-Richtlinien") on LDL-apheresis treatment require the user to justify every two years the indication for this extracorporeal procedure with the local lipid-advisory committee or the health insurance company (1). It is noteworthy, that the number of applications differ from state to state, committee to committee, and from insurance company to insurance company. In some cases detailed laboratory data including the lipid profiles over the last six months have to be submitted to the health insurance companies. Such requirements are an enormous economic and time-consuming burden for the limited laboratory budgets.

On the other hand, because of the rather vague statements of the present German guidelines ("NUB-Richtlinien") (1), it is unclear to the user, when treatment has to be initiated. Clear guidelines regarding for example limits and indices of the LDL-Apheresis procedure, are not currently available. Consequently the health insurance companies can limit the number of LDL-apheresis treatments for economic reasons.

Discussion

On account of the above considerations, we urgently require measures for quality assurance and quality management of LDL-apheresis treatment. The established clinical criteria and recommendations need to be critically reviewed. The message of the reviewed guidelines should be prepared in a clear and easy-to-use form for physicians. Furthermore, as a result of these crucial discussions, an independent LDL-apheresis register should be built up, that documents LDL-apheresis treatments with respect to indication, realization, and quality management.

To address these problems and as a first step to constructing a LDL-Apheresis registry, we initiated a series of consensus conferences starting in 1999, which have taken place every 6 months. The participants at these conferences come from different medical disciplines, including cardiology, nephrology and laboratory medicine and have expertise of atherosclerosis and/or lipidology. All are involved in the indication process and/or the treatment of patients using LDL-Apheresis. The aim of these conferences is to specify the clinical criteria and recommendations for LDL-Apheresis. In addition, these recommendations have been discussed with members of the German Society of Cardiology and the German study group of Clinical Nephrology and approved as an updated recommendation for the indication of LDL-Apheresis:

Recommendations for the indication of treatment with selective extracorporeal plasma therapy (LDL-Apheresis) in the prevention of coronary heart disease.

Indications for LDL-Apheresis:

- All patients suffering from homozygous familial hypercholesterolemia, with functional or genetically determined lack or dysfunction of LDL-receptors and plasma LDL-cholesterol levels > 13.0 mmol/l (>500 mg/dl).
- Patients suffering from coronary heart disease (CHD) documented by clinical symptoms and imaging procedures, in which over a period of at least 3 months the plasma LDL-cholesterol levels can not be lowered below 3.3 mmol/l (130 mg/dl) by a generally accepted, maximal drug-induced and documented therapy in combination with a cholesterol-lowering diet.

- Patients with progression of their CHD documented by clinical symptoms and imaging procedures and repeated plasma Lp(a) levels > 60 mg/dl, even if the plasma LDL-cholesterol levels are lower than 3.3 mmol/l (130 mg/dl).

These main points for the indication of LDL-Apheresis present the basis for the LDL-Apheresis registry. As an urgent request of the consensus conference was the necessity, to provide:
I. Additional information and help for decision making prior to initiation of LDL-Apheresis:

a) additional coronary risk factors such as hypertriglyceridemia, low HDL levels, increased homocysteine are not an indication for LDL-Apheresis treatment at present.
b) if in the scope of hypercholesterolemia, regularly increased fibrinogen levels (>350 mg/dl) are determined and an acute existing inflammatory disease is excluded, a fibrinogen-eliminating LDL-Apheresis system should be preferentially used. Isolated high levels of fibrinogen are not an indication for LDL-Apheresis at present.
c) when on the basis of increased Lp(a) levels an indication for LDL-Apheresis treatment has been assessed, a decrease of at least 60% related to the starting level should be demanded of the LDL-Apheresis procedure. During the extracorporeal therapy a control of plasma Lp(a) levels should be performed every 12th treatment (quarterly or half-yearly).
d) There is no age-related limit for LDL-Apheresis treatment.

II. Additional information regarding LDL-Apheresis treatment:
(1) Demands on the treatment procedure:
after a single LDL-Apheresis treatment the plasma LDL-cholesterol levels should be decreased by at least 60% through this procedure.
(2) Documentation of the treatment procedure:
a) at the beginning of the first LDL-Apheresis treatment, plasma levels of cholesterol, triglycerides, LDL-cholesterol, apolipoprotein B, HDL-cholesterol, Lp(a), and fibrinogen should be determined and documented.
b) during the first 4 treatments cholesterol, triglycerides, LDL-cholesterol, HDL-cholesterol, and Lp(a) should be determined at the start and at the end of each LDL-Apheresis treatment, to assess the appropriate blood or plasma volume for the extracorporeal treatments.
c) from the 4th treatment on only the cholesterol levels at the start of every LDL-Apheresis should be determined; a general lipid profile (including cholesterol, triglycerides, LDL-cholesterol, and LDL-cholesterol) should be obtained at the start and at the end every 12th treatment (quarterly or half-yearly). If necessary, blood or plasma volume for the extracorporeal treatments should be adapted to the actual elimination capacity.
d) with respect to hemodilution effects the HDL-cholesterol levels should not decrease due to extracorporeal treatment. If a single LDL-Apheresis decreases the plasma HDL-cholesterol by more than 20% compared to the starting level, the LDL-Apheresis system used should be changed.
e) in addition to the lipid profiles, coagulation profiles and electrolytes should also be checked regularly.
(3) Quality management
a) at the beginning of extracorporeal therapy and in at least annual intervals a detailed clinical examination of the patients with respect to internal medicine/cardiology should be performed.
b) the clinical results, the findings of therapeutic development and the used Apheresis regimen should be recorded in a central LDL-Apheresis registry. The aim of this registry is a safe and objective evaluation of LDL-Apheresis systems and treatments with respect to clinical efficiency, safety and transparency. Therefore, as a result of this evaluation the treatment quality of an isolated case can be secured.
c) standardized regular clinical and laboratory examinations will be established for the LDL-Apheresis registry.

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Heteromeric Amino Acid Transporters: Structure/Function and Disease

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The heteromeric amino acid transporters (HATs) are composed by a heavy subunit (rBAT or 4F2hc) and a light subunit (LAT-1, LAT-2, y + LAT-1, y + LAT-2, asc-1, xCT, bo,+AT, and two orphan subunits) bound by a disulfide bridge. These light subunits (SLC7A5 to SLC7A11) belong to the large superfamily of APC transporters. Mutations in three genes within this family cause inherited primary aminoacidurias: rBAT (SLC3A1) and bo,+AT (SLC7A9) cause Type I and non-Type I cystinuria, and y + LAT-1 (SLC7A7) cause Lysinuric protein intolerance. To date, The International Cystinuria Consortium have found mutations in rBAT or bo,+AT that explain 80% of the cases of cystinuria studied. Co-immunoprecipitation from kidney revealed that rBAT and bo,+AT form a complex, but not all rBAT is pull down with bo,+AT antibodies. This suggests that an unknown heavy subunit other than rBAT is bound to bo,+AT. In HeLa transfected cells rBAT and bo,+AT co-immunoprecipitate, help each other to reach the plasma membrane and co-express system bo, + amino acid transport activity (exchange between cystine and dibasic amino acids (influx) and neutral amino acids (efflux). Moreover, bo,+AT induces maturation and stabilization of rBAT. Reconstitution into proteoliposomes shows that system bo,+is indeed an exchanger, and that the light subunit bo,+AT is fully active in the absence of rBAT. Study of the transport mechanism of systrem bo,+from chicken brush-border jejunum revealed a sequential mechanism of exchange compatible with a double transport pathway with alternating accessibility. Interestingly, a cystinuria-specific rBAT mutant (R365W)

shows a loss of coupling of the exchange mechanism, and the R265W/bo,+AT-induced system bo,+behaves as an uniport. Arginine 365 is located within the large extracellular domain of rBAT that shows significant homoly to α-glucosidases. Homology modelling of this domain does not give the position of Arg365 with enough accuracy. The 3D structure of this domain will be necessary to understand why the "helping" rBAT subunit affects the transport mechanism of the catalytic bo,+AT subunit.

Crosstalk between Endocytosis and Na^+/H^+-Exchange

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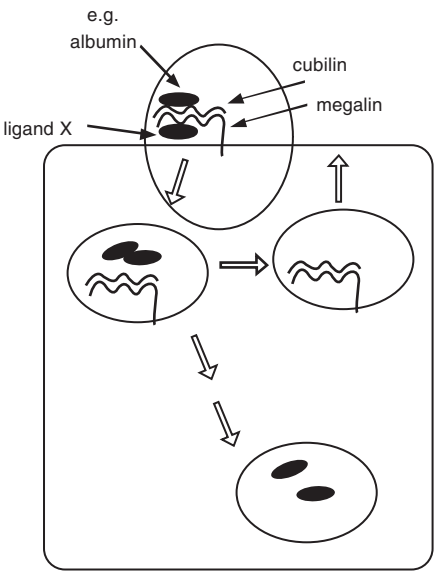
Key Words

Endocytosis, Na^+/H^+-exchange, megalin

Receptor-mediated, clathrin-dependent endocytosis is an essential mechanism for the transport of a variety of macromolecules into cells as well as for antigen presentation, maintenance of cell polarity and regulation of cell-surface protein expression [1]. One example for receptor-mediated endocytosis is the megalin/-cubilin-mediated uptake of filtered proteins across the apical membrane of renal proximal tubular cells [2–6]. In renal proximal tubule the importance of clathrin-mediated endocytosis is threefold: (a) it contributes to vitamin homeostasis and prevents the loss of valuable amino acids, (b) it can induce tubulointerstitial inflammation and fibrosis [7,8] and (c) it regulates membrane protein surface density.

Functional studies on rat kidney indicated that megalin, a 517 kDa monomeric protein in proximal tubular brush-border [9], is involved in the endocytosis of albumin and other filtered proteins [2]. Further studies, in OK-cells as well as in rat kidney, support this hypothesis [6]. Megalin is a polyspecific binding protein with a typical cytosolic NPXY-motif for clathrin-mediated internalisation and with 4 clusters of ligand binding repeats, of which the 2nd cluster seems to be of special importance for ligand binding [10–12]. Originally, megalin had been identified as the primary antigen in Heymann nephritis. Megalin delivers its cargo to early and/or late endosomes (Figure 1) where a pH-dependent dissociation occurs [10]. Thereafter, megalin recycles to the plasma membrane, whereas the ligand may be delivered to lysosomes, as in the case of albumin. This behaviour of megalin is in agreement with several functional data on albumin endocytosis. Besides acting as a polyspecific binding protein, megalin can also act as a membrane anchor for a peripheral membrane protein, namely cubilin [4,13]. The most renown function of cubilin is intestinal reabsorption of intrinsic factor-vitamin B12 [13]. However, cubilin is also expressed in proximal tubular cells, where it remains attached to the outer face of the plasma membrane via its interaction with megalin [13]. Because cubilin serves, like megalin, as a binding protein, there is the possibility that it is also involved in albumin binding. There are recent studies which provide evidence that this is indeed the case [6]. Thus, binding of albumin in proximal tubular cells is, at least in part, mediated by the megalin-cubilin complex.

An important process along the endocytic pathway is the proper pH-homeostasis of endosomal compartments [1,14,15], because it may influence ligand-receptor dissociation, vesicle trafficking,



Possible ligands	
Albumin	Transcobalamin
Transthyretin	Vitamin-D-BP
TBG	Retinol-BP
EGF	Lysozym
Insulin	Cytochrom C
PTH	β_2-Microglobulin
Prolactin	

Fig. 1. Scheme of the megalin/cubilin-complex in the apical membrane of proximal tubular cells. This scavenger complex supports the endocytic reabsorption of a variety of ligands, some of which are listed in the right panel. The receptor-ligand complex enters endocytic vesicles and the early endosomal compartment. There, the ligand dissociates and is directed in most cases to lysosomes for degradation. The megalin/cubilin-complex recycles to the apical membrane.

endosomal fusion events, recycling to the plasma membrane and COP-coat formation [1,14,16–18]. pH-homeostasis is maintained, at least in part, by the vacuole-type H^+-ATPase [19]. In addition, evidence was presented for the involvement of another proton transporter, namely Na^+/H^+-exchange-3 (NHE-3), in endosomal pH-homeostasis [20–22]. NHE-3 is one of five plasma membrane Na^+/H^+-exchangers and is expressed in the apical membrane of renal proximal tubule and intestinal epithelial cells [23], where it is important for fluid, sodium and bicarbonate reabsorption [24].

In cells expressing NHE-3, this transporter cycles between the apical plasma membrane and the early endosomal compartment [25,26], with more than 50% of transporter protein residing in early endosomal compartments, the majority most probably in recycling endosomes [21,27] (Figure 2). NHE-3 is internalised through the clathrin-mediated pathway which depends on the integrity of the actin cytoskeleton, similar to a variety of receptors serving in receptor-mediated endocytosis [28]. In addition, Biemesderfer *et al*. showed that megalin and NHE-3 can interact specifically in proximal tubular cells [29]. Apical membrane NHE-3-activity is subject to a variety of regulatory events, as for

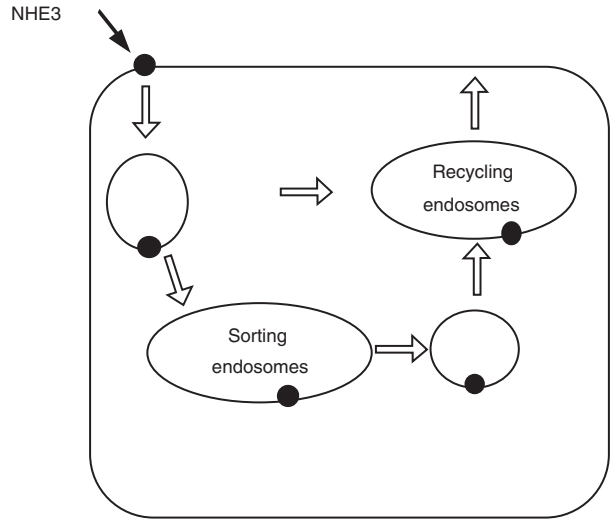


Fig. 2. The recycling pathway of NHE-3. The intracellular "storing site" are most probably recycling endosomes.

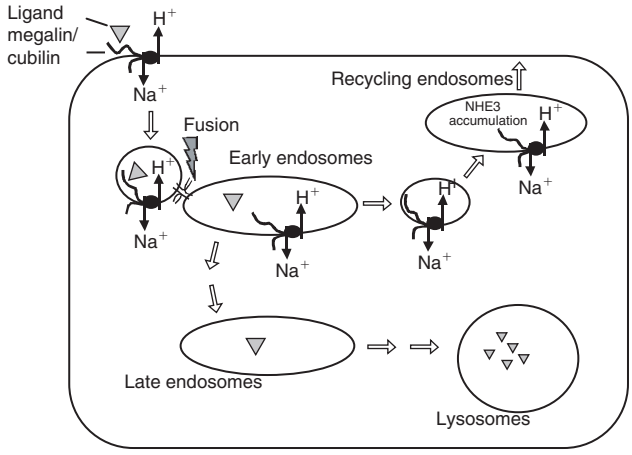


Fig. 3. Hypothetical model for the crosstalk between NHE-3 and the megalin/cubilin-complex. Both travel side-by-side through the early endosomal compartment. The megalin/cubilin-complex carries the cargo (ligand), whereas the NHE-3 facilitates travelling and delivery by supporting vesicle fusion and vesicular pH-homeostasis.

example inhibition by cAMP-dependent kinase [30], PTH [31], G-proteins [32] or stimulation by phosphatidylinositol 3′-kinase [26] and PKC [33]. Some of these stimuli exert their regulatory action by a modulation of endo-/exocytosis equilibrium of NHE-3 (e.g. phosphatidylinositol 3′-kinase and PKC).

In summary, the NHE-3 travels along the clathrin-dependent endocytic pathway and is, at least in part, functional along this pathway. Thus, the question arises here, whether only endocytosis influences NHE-3 or whether NHE-3 may also influence clathrin-dependent endocytosis. Recently, we showed that reduced activity of NHE-3 in renal proximal tubular OK- or $LLCPK_1$-cells leads to disturbed endosomal pH-homeostasis, a dramatic reduction in receptor-mediated endocytosis of albumin and reduced endocytic vesicle fusion activity [5,34]. These effects could not be attributed to an inhibition of plasma membrane NHE-3 but only to an inhibition of endosomal membrane NHE-3.

In conclusion, there seems to be a mutual modulation between clathrin-dependent endocytosis and NHE-3 (Figure 3). Clathrin-dependent endocytosis represents one leg of the endo-/exocytosis equilibrium of NHE-3, whereas at the same time NHE-3 supports certain events along this endocytic pathway (vesicle pH-homeostasis, vesicle fusion). Thus, there exists a complex crosstalk between endocytosis and NHE-3 which may also be involved in the regulation of clathrin-dependent endocytosis.

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Primary Hypertension – A Renal Disease?

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Key Words

Hypertension, Renal transplantation, Spontaneously hypertensive rat

Summary

The results of experimental renal cross-transplantation experiments between genetically hypertensive and normotensive rat strains show that hypertension travels with the kidney. Many of these studies were performed with spontaneously hypertensive rats (SHR). Recipients of an SHR kidney show increased renal sodium retention but no activation of the renin-angiotensin or sympathetic nervous system. Using a normotensive congenic strain that was developed for histocompatibility with SHR it has recently been shown that the maintenance of hypertension in SHR critically depends on the presence of a hypertensive kidney. Microsurgical transplantation techniques involving congenic or consomic animals allow for kidney specific gene transfer and to determine the contribution of specific genes to the genetically determined capability of the kidney to induce primary hypertension.

Introduction

Quantitative analyses of physiological systems involved in arterial pressure regulation revealed that isolated changes in total peripheral resistance or cardiac output do not induce sustained hypertension unless the renal capacity to excrete electrolytes and fluid at a given arterial pressure level is compromised simultaneously [1]. Increased renal retention of sodium and fluid causes arterial pressure to rise which restores body sodium and fluid balance via the pressure natriuresis and diuresis mechanism [1]. These findings which were the results of animal experiments and mathematic modeling contributed to direct attention to renal physiology and pathophysiology in the field of hypertension research.

Various rat models of genetic hypertension have been established for experimental investigation of hypertension genetics and pathophysiology including spontaneously hypertensive rats of the Okamoto-Aoki strain (SHR), Dahl salt-sensitive rats, Milan hypertensive rats, Lyon hypertensive rats, Prague hypertensive rats. Spontaneously hypertensive rats of the Okamoto-Aoki strain have been the most frequently used experimental animals in research on genetics and pathology of arterial hypertension. Several pathophysiological features of this rat strain are important for the understanding of human hypertension and for preclinical development of antihypertensive drugs.

Already at the age of 3–6 weeks the pressure natriuresis and diuresis relationship in these animals is shifted to elevated arterial pressure levels [2]. Renal afferent arteriolar resistance and tubular sodium reabsorption are increased compared to normotensive animals and a putative renal incretory blood pressure lowering mechanism is reset to elevated arterial pressure levels. These abnormalities in renal function are consistent with an involvement of renal mechanisms in the pathophysiology of hypertension in SHR.

In addition to intrarenal mechanisms neuroendocrine factors may contribute to the development of arterial hypertension in

SHR. Sympathetic nerve activity is elevated in this strain and sympathetic innervation of several target organs develops faster and is more dense than in normotensive rats. Neurohumoral reactivity to environmental stress is enhanced compared to normotensive rats. Brief angiotensin converting enzyme inhibition in juvenile SHR as well as neonatal interruption of peripheral sympathetic innervation reduce arterial pressure long-term associated with a reduction in peripheral vascular resistance [3,4]. These effects may be at least in part due to interference with renal development and function.

Blood Pressure Travels with the Kidney in Renal Cross-transplantation Experiments

In order to investigate the contribution of renal mechanisms to the development and maintenance of primary hypertension renal cross transplantation experiments were performed. This type of experiments requires genetically hypertensive and normotensive rat strains with good histocompatibility to avoid confounding effects of secondary hypertension due to chronic renal allograft rejection and/or immunosuppression.

It has been demonstrated in Dahl salt sensitive rats [5], Milan hypertensive rats [6] and in Prague hypertensive rats [7] that arterial hypertension can be transferred with a renal graft from either hypertensive strain to normotensive histocompatible recipients. Furthermore, renal grafts from the respective normotensive control strains lowered arterial pressure in these three genetically hypertensive rat strains. Thus, it has been consistently shown that renal mechanisms play a major role in the maintenance of genetic forms of hypertension in rats.

Most Experimental Renal Cross-transplantation Studies were Done in SHR

In the past, investigations on renal mechanisms in SHR genetic hypertension applying renal transplantation techniques have been more circumstantial than in other genetically hypertensive rat strains [5–7] because no histocompatible normotensive rat strain was available for cross transplantation experiments. Therefore F1-hybrids ($F1H_{(SHRxWKY)}$) derived from intercrossing SHR and normotensive inbred Wistar-Kyoto rats (WKY) have been used as recipients for both SHR and WKY kidneys. $F1H_{(SHRxWKY)}$ are heterozygous at all autosomal gene loci. Arterial pressure in these animals is significantly less than in SHR but somewhat elevated when compared to WKY. Histocompatibility genes of both parental strains are coexpressed in these animals allowing for transplantation of renal grafts from either parental strain. However, $F1H_{(SHRxWKY)}$ cannot be used as kidney donors for SHR recipients. When $F1H_{(SHRxWKY)}$ were transplanted with an SHR kidney and both native kidneys were removed, recipients developed arterial hypertension [8]. Transplantation of an $F1H_{(SHRxWKY)}$ or WKY kidney into $F1H_{(SHR\ x\ WKY)}$ did not induce hypertension [8–10].

An important question regarding the role of the kidney in long-term arterial pressure regulation in SHR is if arterial pressure can be lowered by a kidney graft from genetically normotensive donors. An initial study addressed this issue by performing renal allotransplantation [11]. At the time of that study [11] no normotensive donor strain with the SHR haplotype of the major histocompatibility complex was available. SHR were either transplanted with an SHR kidney or they received a renal allograft from a normotensive donor strain unrelated to SHR. To prevent allograft rejection recipients were treated with an anti-CD4 antibody and cyclosporine. The same treatment was administered to controls transplanted with a renal isograft. Six weeks after transplantation, arterial pressure was lower in SHR transplanted with a kidney from normotensive donors than in SHR transplanted with an SHR kidney but significantly higher than in controls of the normotensive donor strain with a renal isograft. This experiment demonstrated that in SHR the maintenance of hypertension depends to a certain extent on intrarenal mechanisms [11]. However, this experiment did not allow to estimate the quantitative contributions of extrarenal and renal mechanisms to the level of arterial pressure in SHR because of confounding effects of chronic allograft rejection and immunosuppression which per se may induce elevated arterial pressure.

In order to exclude confounding effects of allograft rejection and immunosuppression on long-term arterial pressure a normotensive histocompatible rat strain was established by a breeding strategy leading to congenic animals. These normotensive BB.1K rats are homozygous for a 2 cM segment of SHR chromosome 20 including the class Ia and class II genes of the SHR major histocompatibility complex and allow for cross transplantation experiments in SHR without the need of immunosuppression. We could demonstrate that a solitary BB.1K kidney transplanted into bilaterally nephrectomized SHR lowered arterial pressure to the level of the normotensive donor strain (Figure 1) accompanied by improved renal sodium excretion and regression of left ventricular hypertrophy [12]. These data indicate that renal mechanisms are central for the maintenance of arterial hypertension in SHR.

Recipients of an SHR Kidney Show Increased Renal Sodium Retention but no Activation of the Renin-angiotensin or Sympathetic Nervous System

A consistent observation of several experiments was increased renal sodium retention in recipients of an SHR kidney compared to controls transplanted with a WKY kidney associated with suppressed aldosterone secretion [9]. Data on the renin angiotensin system did not suggest that its activation is a major contributor to the development of renal post-transplantation hypertension in recipients of an SHR kidney. Plasma renin activity and plasma

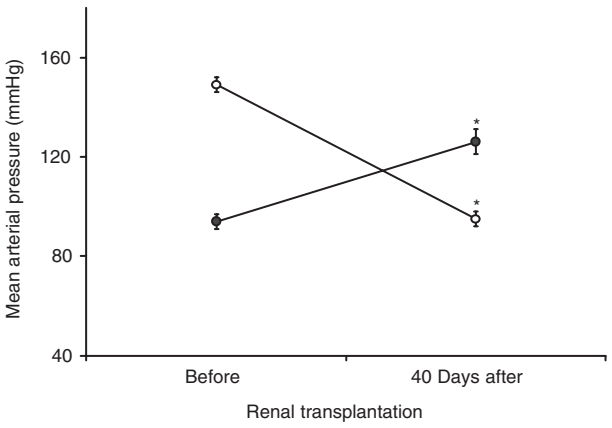

Fig. 1. Mean arterial pressure in congenic normotensive rats (BB.1K) ($n = 7$) before and after bilateral nephrectomy and transplantation of an SHR kidney (closed circles) as well as in SHR ($n = 5$) before and after transplantation of a BB.1K kidney (open circles). * $p < 0.01$ vs. controls

angiotensin converting enzyme activity as well as plasma angiotensin I and angiotensin II concentrations were similar in recipients of an SHR and a WKY kidney [13]. Intrarenal renin activity [8] as well as mRNA contents for renin, angiotensin converting enzyme and angiotensinogen [13] were almost identical in transplanted SHR and WKY kidneys.

Sympathetic reinnervation of SHR kidney grafts does not contribute to renal post-transplantation hypertension. Measurements of adrenal tyrosine hydroxylase mRNA contents and analyses of discharge characteristics of splanchnic sympathetic nerves did not reveal sympathetic activation to be associated with hypertension in recipients of an SHR kidney [10]. Responses of sympathetic nerve activity and arterial pressure to a centrally acting sympatholytic substance [10] did not provide evidence for elevated central sympathetic drive and increased dependence of arterial pressure on sympathetic tone in recipients of an SHR kidney. Thus, current data do not support the hypothesis that neurohormonal activation is of major importance for the development of renal post-transplantation hypertension.

Outlook

Sophisticated experimental animal breeding strategies and increasing possibilities of molecular genetic characterization of rat strains has led to a rising number of congenic and consomic rat lines available for hypertension research [14]. In these inbred animals defined genome fragments of "normotensive" origin have been exchanged by corresponding parts of the genome of a hypertensive inbred rat strain and vice versa. Microsurgical transplantation techniques involving congenic or consomic animals allow for so called kidney specific gene transfer [15]. Thus, it will be possible to characterize in greater detail what genes contribute to the genetically determined capability of the kidney to induce primary hypertension.

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Renal Ischemia-Reperfusion Injury: Is Apoptosis Involved?

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Key Words

Apoptosis, kidney, ischemia-reperfusion, inflammation

Introduction

Ischemia followed by reperfusion leads to severe organ injury and dysfunction. Renal ischemia-reperfusion (I/R) induced organ injury is seen after renal transplantation, major abdominal surgery, vascular surgery, coronary bypass surgery, in trauma, and sepsis. The pathophysiology of I/R injury is characterized by early cellular dysfunction and cell-death. Initial cellular damage eventually leads to an intense inflammatory response, which is considered to be the most important cause of tissue injury and organ failure upon I/R. Necrosis is believed to be the major form of cell death involved in I/R injury. However, evidence is accumulating that apoptosis plays an essential role in the pathogenesis of renal I/R injury [1–9].

Induction and Execution of Apoptosis upon I/R

Apoptosis can be induced by death-receptor dependent (extrinsic) or death-receptor independent (intrinsic) stimuli. The death-receptor dependent pathway is triggered by the binding of extracellular factors, such as TNF-α and Fas-ligand (CD95L), to so called death-receptors, respectively TNF-Receptor 1 (CD120a) and Fas. Binding of ligand to receptor induces clustering of the intracellular receptor-coupled death-domains. These recruit adapter proteins TRADD (TNF-receptor associated death domain) or FADD (Fas-associated death domain), both containing a death effector domain (DED), forming a death inducing signalling complex (DISC). In the DISC multiple procaspase-8 molecules are recruited from the cytoplasm resulting in activation of caspase-8. Activated caspase-8 initiates the cleavage cascade of downstream caspases, such as caspase-3,

which leads to the execution of apoptotic cell-death. In vivo, TNF-α as well as Fas-ligand have been implicated in the induction of apoptosis after renal I/R [9–12].
The death-receptor independent pathway is mainly localised to the mitochondria. A characteristic of this pathway is the increased mitochondrial permeability and release of cytochrome-c from the mitochondrion, which can be initiated by stimuli such as hypoxia and oxygen free radicals. In the presence of ATP, cytochrome-c released from the mitochondrion associates with the extramitochondrial protein Apoptosis Protease Activating Factor-1 (APAF-1). Conformational changes lead to exposure of a caspase recruitment domain (CARD) which recruits procaspase-9, forming the apoptosome complex, consequently leading to activation of procaspase-9. Activated caspase-9 further activates downstream caspases like caspase-3, resulting in progression to the execution phase of apoptosis. The intrinsic pathway is involved in hypoxia-reoxygenation injury in vitro, among others in cultured kidney cells [13–16]. Little is known about the functional role of the intrinsic pathway in the development of I/R injury in vivo.
After induction, the execution of apoptosis is performed by caspases, a group of intracellular cysteine proteases, which cleave their substrates behind specific aspartic acid residues. Caspases are constitutively present as inactive pro-enzymes. First, initiator caspases (caspase-8 and caspase-9) are activated, which in turn leads to activation of so-called effector caspases (caspase -3, -6, and -7). These caspases activate specific substrates, which among others dismantle the apoptotic cell and degrade the DNA. Finally, the apoptotic cell fragments are phagocytosed by neighbouring cells or macrophages. Activation of caspases in experimental I/R injury has been documented in several models. Recently, we have shown that inhibition of apoptosis with a specific caspase-inhibitor is strongly protective against renal I/R injury, suggesting that apoptosis is essentially involved in the pathophysiology of I/R injury [1].

Regulation of Apoptosis after I/R

The apoptotic process, deciding between cell-survival and cell-death, has to be closely regulated, and several regulating factors, extra- as well as intracellular, have been described. The activation of caspases is regulated by several mechanisms. The cytoplasmatic protein cFLIP (cellular FLICE inhibitory protein) is a competitive inhibitor of caspase-8, regulating activation of this initiator caspase and thereby preventing uncontrolled cell-death. In our model of renal I/R, evident depletion of cFLIP already starts during ischemia [unpublished data]. Caspase-9 activation is closely regulated by pro- and anti-apoptotic proteins from the Bcl-2 family. Other intracellular proteins involved in regulation of apoptosis are the IAPs (inhibitors of apoptosis proteins) and Smac/DIABLO (IAP-inhibitor).
Recently, in vitro work has shown that growth factors, such as Insulin-like Growth Factor (IGF-1), Epidermal Growth Factor (EGF) and Hepatocyte Growth Factor (HGF) are also involved in the regulation of apoptosis and should be regarded as survival-factors. In vivo studies show that these survival-factors regulate I/R-induced apoptosis and subsequent inflammation [1,15].

The Role of Apoptosis in I/R Induced Inflammation

Normally, apoptotic cells become phagocytosed by macrophages or neighbouring cells, without provoking an inflammatory response. Thus, in contrast to necrotic cell death, apoptotic cell death is paradigmatically thought to be a non-inflammatory event. However, evidence is rising that apoptosis contributes to the development of an inflammatory response upon I/R [1]. Pathological situations like I/R injury may lead to massive apoptosis, in contrast to that seen under physiological circumstances. When the clearance capacity of phagocytosing cells is overwhelmed by the amount of apoptotic cells secondary necrosis of apoptotic cells occurs, which may subsequently result in an inflammatory response. Activated caspases also have pro-inflammatory capacities. Caspase-1 (ICE, interleukin-1β-converting enzyme) is involved in processing pro-IL-1β and pro-IL-18, both of which have been implicated in the development of inflammation in renal I/R injury [17]. However, in our hands, caspase-1 knockout mice were only partially protected against renal I/R injury, suggesting that caspase-1 has a limited role in the development of I/R injury [18]. Caspase-7 has been demonstrated to be involved in renal I/R and to process the chemokine EMAP-II, which is known to attract neutrophils, a key-feature of the inflammatory response in renal I/R injury [1]. We also observed EMAP-II activation and up-regulation of the chemokines KC and MIP-2 in our model of renal I/R, a process which could be prevented by inhibition of apoptosis [1,19]. Thus, caspase activation may not only induce apoptosis in renal I/R, but may be functionally involved in the development of the inflammatory response as well. In line, inhibition of caspase activation with a specific inhibitor in a murine model of renal I/R not only prevents initial apoptosis, but also diminishes the subsequent inflammatory reaction [1].

Conclusion

Recent studies in experimental renal I/R injury have provided new insights into its pathophysiology, addressing a functional role for apoptosis. This insight has led to the use of anti-apoptotic agents, which have appeared to be remarkably efficient in preventing I/R injury in experimental models.

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Pathology of Experimental Renal Transplants Lessons for Human Renal Transplantion

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Renal transplantation in the rat is often used as an experimental model for allogeneic transplants in man. Acute vascular and interstitial rejection can be observed in different severities depending upon the MHC disparity and the strain combinations. The morphologic and functional phenomena appear to be equivalent to those observed in human renal transplants. Ureteral obstruction in the rat will initiate and accacerbate interstitial and vascular rejection of an allogeneic renal transplant. This has helped make clear the tantamount importance of avoiding reflux nephropathy in the interpretation of transplant studies. It is doubtful whether this important caveat has been rigorously observed in all published studies on rat renal transplants.

Chronic renal transplant dysfunction is a major complication of long term human renal transplants. Arterial hypertension, dyslipidemia and smoldering rejection may contribute to chronic renal transplant dysfunction. It is characterized by several morphologic phenomena e.g. chronic vascular rejection, degenerative vascular disease with subintimal fibrosis, transplant glomerulopathy in many different phentotypes, interstitial fibrosis and chronic tubular damage, and mononuclear infiltrates of the interstitium with varying subpopulations of leucocytes. No single rat transplantation model has been developed that will faithfully imitate these phenomena. However, two models which appear to at least partly imitate chronic renal transplant dysfunction have been proposed (Kunter *et al*, in preparation). The first model is the transplantation of a Fisher rat kidney into a Lewis rat. After a period of 8–16 weeks one can observe focal, glomerular and tubulointerstitial lesions reminiscent of chronic transplant dysfunction phenomena in human renal transplants. Although some reports state otherwise, in our view it is important not to use any immunosuppression to observe these phenomena. The second model is the transplantation of a Dark Agouti (DA) kidney into a Wistar Furth (WF) rat with consecutive cyclosporin A therapy for 10 days. This model imitates chronic vascular rejection but is less suitable for studying the chronic glomerular and tubulo-interstitial damage.

We have been specifically interested in the identification of non-immunologic factors as modulators of acute rejection and consecutive chronic renal damage. Three factors that have been found to have a major impact on endothelial function and the interaction of endothelial cells with mononuclear cells have been characterized in acute transplant rejection models in the rat. These are:

1. nitric oxide (NO)
2. transcription factor NFκB,
3. Chemokines/Chemokine receptors

1. NO

Nitric oxide has several functions that merit its classification as a protective agent for endothelial cells. It is a vasodilator; it contributes to the non-thrombogenic surface of endothelial cells; it decreases the proliferation of vascular smooth muscle cells; it helps to maintain the permeability barrier of endothelial cells, and it decreases production of extracellular matrix. On the other hand, NO in high concentrations can be cytotoxic and may induce apoptosis. We have found that unselective NO synthase blockade can lead to a dramatic decrease of allograft survival by aggravation of the allo-immune-response. The stimulation of endothelial NO-synthesis by supplementation of the transplanted animal with L-arginine was found to induce an increase in glomerular filtration rate and renal blood flow as well as a reduction of vascular injury and tubulo-interstitial rejection. In addition, endothelial NO-synthesis was improved and production of superoxide radical by NO-synthase was reduced. Supplementation of the transplant recipient with L-sepiapterin, a precursor of tetrahydrobiopterin (BH_4), was found to inhibit intrarenal generation of superoxide anion as well as monocyte influx for 24 hours following transplantation. This benifical effect of L-sepiapterin is thought to be due to prevention of NO-synthase uncoupling.

In contrast, the inhibition of inducible NO-synthase in infiltrating mononuclear cells by specific inducible NO-synthase inhibitors was found to improve renal graft haemodynamics and to significantly diminish tubulo interstitial injury as well as nitrotyrosine modification of tubular epithelial and mononuclear cells that had infiltrated the renal interstitium. This suggests that the high level of NO generated by inducible NO-synthase in monocytes is directly involved in acute rejection probably by the generation of peroxinitrite [1,2,3].

2. NFκB

The transcription factor NFκB may be an important player in reperfusion injury and rejection. The transcriptional activation of

the genes that encode the adhesion molecules essential for the transendothelial migration of inflammatory cells, is regulated in part, by induced transcription factors such as NFκB. Oxygenation of an allograft can cause the release of reactive oxygen species and tumor necrosis factor-α. The translocation of the heterodimeric NFκB-complex from the cytoplasm to the nucleus can be induced by inflammatory cytokines such as tumor necrosis factor-α and by the redox status of the cell. Oxidative stress will increase nuclear NFκB-activity. We evaluated the use of a NFκB decoy strategy in allogeneic kidney transplantation (Brown Norway rat kidney to Lewis rat recipients). We found that perfusion of the renal allograft with an NFκB decoy oligonucleotide prior to transplantation reduced nuclear NFκB activity *in vivo*, inhibited expression of adhesion molecules in the endothelium of the graft, and led to a significant reduction of periarterial infiltration by monocytes and macrophages [4]. This sort of decoy approach might be a useful tool to reduce acute monocyte infiltration in renal allografts.

3. Chemokines/Chemokine Receptors

The recruitment of leukocytes from the peripheral circulation into the transplanted organ involves a complex interplay between a series of molecules expressed on leukocyte and endothelial surfaces. Chemokines, a large superfamily of structurally related cytokines have been shown to selectively promote the rapid adhesion, chemotaxis, and activation of specific leukocyte effe tor subpopulations. The chemokine RANTES/CCL5, a member of the C-C chemokine subfamily, is a potent chemoattractant for T cells, monocytes, natural killer cells, basophils, and eosinophils. RANTES is a ligand for a number of chemokine receptors including CCR1, CCR3, CCR5 and DARC (Duffy antigen receptor for chemokines) in humans.

We investigated the functional role of RANTES and its receptors in rat models of acute renal allograft rejection. Modification of the amino terminus of the RANTES protein can dramatically alter its properties. The addition of a single methionine residue changes the agonist protein into a RANTES receptor antagonist with nanomolar potency. This antagonist, Met-RANTES, is bioactive in mouse and rat (Proudfoot A.E., unpublished results), and has been shown to suppress inflammation in murine models of allergic skin and rheumatoid arthritis and to partially inhibit necrotizing glomerulonephritis.

In a renal transplant model (Fisher $RT1^{(v)}$ rat kidney into Lewis $RT1^{l}$ rat) without using any additional immune suppressant, Met-RANTES-treated animals showed a significant reduction in vascular injury score and tubular damage relative to untreated animals. In a severe rejection model (Brown-Norway kidneys into Lewis rat) Met-RANTES significantly augmented low-dose cyclosporin A treatment to reduce all aspects of renal injury including interstitial inflammation [5].

In summary, the rat allogeneic renal transplant can be used to elucidate the immunulogic and non-immulogic factors that contribute to acute and chronic renal transplant rejection. Specific transplantation models or rat strain combinations can be used to achieve specific phenomena that mirror pathophysiological events in human transplant nephropathy.

Studies in the rat have clearly demonstrated that non-immunologic factors: reactive oxygen species, transcription factors and chemokines/chemokine receptors, play essential roles in modulation of acute, and probably chronic dysfunction phenomena.

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