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Alterations in Purinoceptor Expression in Human Long Saphenous Vein during Varicose Disease

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Objectives. *Varicose veins are dilated tortuous veins of varying tone. Purinergic signalling is important in the control of tone and in mediating trophic changes in blood vessels. The expression of P2 receptors in control and varicose veins will be examined.*
Methods. *Purinergic signalling in circular and longitudinal smooth muscle of the human long saphenous vein was studied in control and varicose tissues using immunohistochemistry, organ bath pharmacology and electron microscopy.*
Results. *$P2X_1$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors were present on circular and longitudinal smooth muscle. Purine-mediated circular and longitudinal muscle contractions were weaker in varicose veins. Electron microscopy and immunohistochemistry findings support the view that smooth muscle cells change from the contractile to synthetic phenotype in varicose veins, associated with an upregulation of $P2Y_1$ and $P2Y_2$ receptors and a down regulation of $P2X_1$ receptors.*
Conclusions. *Down regulation of $P2X_1$ receptors on the smooth muscle of varicose veins is associated with loss of contractile activity. Upregulation of $P2Y_1$ and $P2Y_2$ receptors is associated with a shift from contractile to synthetic and/or proliferative roles. The phenotype change in smooth muscle is associated with weakening of vein walls and may be a causal factor in the development of varicose veins.*

Keywords: P2X receptor; P2Y receptor; Human; Saphenous vein; Phenotype.

Introduction

The long saphenous vein (LSV) is the most widely used autogenous venous graft due to its thick walls, free availability and being the longest in the body. However, it commonly becomes varicose and, due to chronic venous insufficiency, leads to venous ulceration.[1] It is a three-layered structure with an intima, media and outer adventitia. Varicose perivascular nerve fibres lie at the adventitia-medial border and form neuroeffector junctions. The smooth muscle cells (SMC) are in electrical continuity with each other via gap junctions.[2]

Varicose veins are dilated, tortuous, thickened veins, with wall structures varying from hypertrophic to atrophic. In atrophic regions the medial SMC and extracellular matrix (ECM) are diminished, leaving a thin media lying on the adventitial fibrous tissue.[3] In hypertrophic regions the organisation is disturbed; smooth muscle bundles lose their longitudinal and circular direction, fibrous tissue accumulates, and increases in ECM and vasa vasorum occur. There is both hyperplasia and hypertrophy of the intimal SMC.[3,4] These changes are consistent with a change from a contractile to a synthetic phenotype.[3] There is also evidence that SMC have taken up a phagocytic role.[4]

Varicose veins have incompetent venous valves, but an inherent weakness of the muscle wall due to defective smooth muscle and connective tissue metabolism is a contributing factor resulting in vessel dilatation and separation of valve leaflets.[1,5] Similar structural, biochemical and functional changes in varicose tributaries and in non-varicose veins from the same patient[6] suggested that abnormalities within the vein wall exist before the varicosities develop.

Purinergic signalling, where purine nucleotides and nucleosides act as extracellular messengers, have important roles within the cardiovascular

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system.[7] Purines are cotransmitters with noradrenaline (NA) mediating vasoconstriction. Long term (trophic) changes of SMC and endothelial cells also occur.[8] Receptors to purines and pyrimidines are divided into P1 (adenosine) receptors and P2 (purine/pyrimidine) receptors.[9] P2 receptors are subdivided into seven $P2X_{(1-7)}$ ligand-gated ion channel receptors and eight $P2Y_{(1, 2, 4, 6, 11, 12, 13, 14)}$[10] receptors coupled to G proteins.[7,11] The $P2Y_{12}$ receptor antagonist, clopidogrel, is an antithrombotic drug, and adenosine acts on A_1 receptors on the atrioventricular node inhibiting supraventricular tachycardia.[12,13]

Purinergic signalling has been demonstrated in the LSV.[14,15] Purine-mediated contractions on varicose veins were compared with veins from atherosclerotic legs,[16] although not with healthy LSV. Previous pharmacological studies have only examined the circular muscle. Immunohistochemical studies have described the presence of longitudinal muscle but its contractile function is unknown.[17] This study aimed to examine the expression of P2 receptor subtypes in healthy and varicose human LSV and compare the contractile effects mediated by these receptors in circular and longitudinal smooth muscle. Vein wall ultrastructure, with particular emphasis on smooth muscle phenotype, was also studied.

Patients

Proximal end varicose vein segments were obtained from 31 patients (19 female, 12 male, aged 21–77, mean age 46.8 ± 2.6 years) undergoing stripping of their LSV. The most proximal end of the sample was obtained prior to insertion of the stripper. Reflux was confirmed by hand held doppler or venous duplex scanning. Healthy control vein was obtained from 34 patients (6 female, 28 male, aged 41–84, mean age 63.6 ± 1.6 years) undergoing coronary artery bypass surgery. A segment from the proximal end of the LSV was excised. Diabetic patients and control patients with reflux were excluded. Ethics approval was obtained by the joint UCL/ULCH Ethics Committees on Human Research and by the Royal Free Hampstead Research Ethics Committee.

Methods

Immunohistochemistry

Samples were collected in Hanks balanced salt solution (HBSS; Invitrogen, Paisley, UK) and frozen in precooled isopentane. Cryostat cut sections (10 μm) (Reichert Jung CM1800) were collected on gelatine-coated slides, air dried at room temperature, fixed for 4 min in 4% formaldehyde in 0.1 M phosphate buffer solution (PBS) containing 0.2% picric acid, then washed (3×5 min) with PBS. Sections were blocked for 60 min in 10% normal horse serum (NHS) in 0.1 M PBS, containing 0.05% merthiolate. Sections were incubated overnight with two primary antibodies: polyclonal P2X ($P2X_{1-6}$) (Roche Palo Alto, CA, USA) or P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$) (Alomone Laboratories, Jerusalem, Israel) antibodies (1:50–1:200), and monoclonal anti α-smooth muscle actin antibody (Sigma Chemical Co., Poole, UK; 1:400) in 10% NHS in PBS with 0.05% merthiolate. On day 2, sections were washed (3×5 min) in PBS and stained with the secondary antibodies: donkey anti-rabbit Cy3 (Jackson Immunoresearch Laboratories, West Grove, USA; 1:300) and donkey anti-mouse FITC (Jackson; 1:200) in PBS-merthiolate for 60 min. Sections were washed (3×5 min) and mounted in Citifluor (Citifluor Ltd, London, UK). Preabsorption control experiments were performed with the corresponding peptide.

Semi-quantitative assessment of the changes in immunofluorescent intensity were performed by an independent observer, blinded from the patient group from which samples were taken.

Haematoxylin and Eosin (H&E) slides were prepared by fixing in 4% paraformaldehyde in PBS for 10 min. Sections were washed in distilled water then stained for 20 min in Ehrlichs Haematoxylin. Following washing in running tap water, slides were dipped in acid alcohol and washed again for 15 min. Sections were then stained in Eosin for 5 min, dipped in tap water, then washed for 1 min in 70% alcohol, 3 min 100% alcohol, another 3 min 100% alcohol, dried for 3 min in xylene, and finally another 5 min in xylene. Sections were mounted in Eukitt.

Staining was photographed using a Zeiss Axioplan light microscope (Zeiss, Oberkochen, Germany) mounted with a Leica DC 200 digital camera (Leica, Heerbrugg, Switzerland).

Electron microscopy

Vein segments were collected and transported in HBSS then fixed in 2% paraformaldehyde, 2% glutaraldehyde in 0.1 M phosphate solution. Tissue was washed in phosphate buffer, post-fixed in 1% osmium tetroxide in phosphate buffer, en-block stained with a 2% solution of uranyl acetate in distilled water, dehydrated in graded ethanols and embedded in an agar resin. Sections (80 nm) were cut and collected on thin films, counterstained with lead citrate and viewed in a Jeol 1010 TEM.

Pharmacology

Tissues were collected in Krebs solution (pH 7.2; mM): NaCl 133, KCl 4.7, NaH_2PO_4 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, $CaCl_2$ 2.52 and glucose 7.8. Connective tissue was removed and the vessels mounted horizontally to examine circular muscle (by inserting 2 tungsten wires through the lumen, one attached to a support the other to a Grass FTO3C transducer) or vertically to examine longitudinal muscle (by using a 15 mm length of vein, suspended with silk thread) in 10 ml organ baths containing Krebs solution, gassed with 95% O_2–5% CO_2, and maintained at 37 ± 1 °C. Rings were placed under an initial tension of 2 g and longitudinal muscle 2.5 g and allowed to equilibrate for 60 min. Mechanical activity was recorded using PowerLab Chart for Windows (version 4; ADInstruments, Australia). NA (10^{-8} to 10^{-3} M) was applied cumulatively. ATP (10^{-6} to 10^{-3} M) and α,β-methylene ATP (α,β-meATP; 10^{-8} to 10^{-5} M) were applied non-cumulatively, separated by 15 min intervals, washing in between. Increasing concentrations of KCl (10^{-5} to 3×10^{-1} M) were also applied.

On separate segments the effect of P2Y receptor agonists was examined cumulatively. 2-Methylthio ADP (2-MeSADP; 10^{-8} to 10^{-5} M), repeated in the presence of MRS2179 (10^{-6} M); UDP (10^{-6} to 10^{-3} M), repeated in the presence of cibacron blue 3GA (10^{-4} M); UTP (10^{-6} to 10^{-3} M) repeated in the presence of suramin (10^{-4} M). All antagonists were incubated for 45 min.

Electrical field stimulation (100 V, 1–6 Hz, 0.1 msec duration for 1 min every 10–20 min) of longitudinal muscle was tested using platinum wire electrodes, one in the lumen and one outside the vessel, via a Grass SD9 stimulator and frequency response curves were constructed in the absence and presence of prazosin (10^{-6} M) alone, prazosin and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS; 3×10^{-5} M) together and finally in the presence of tetrodotoxin (TTX; 10^{-6} M). All agonists were equilibrated for 30 min.

The integrity of the endothelium was examined in control and varicosed samples by their ability to relax to acetylcholine (ACh). Vessels were precontracted with NA (EC_{50} concentration) and increasing concentrations of ACh were applied (10^{-9}–10^{-3} M).

Statistical analysis

Concentration-response curves were prepared using the software Prism 3.0 (GraphPad Software, Inc., San Diego, CA, USA). Significance was tested using a two-way analysis of variance (ANOVA) followed by a *post hoc* test (Bonferroni's). A probability of $P < 0.05$ was taken as significant.

Results

An un-paired two-tailed *t*-test revealed a significant age difference between the two groups ($P < 0.0001$), the control group being older.

Histology

Sections stained with H&E confirmed previous findings of smooth muscle variation in varicose sections between atrophic and hypertrophic segments.[3] Atrophic sections had reduced ECM and SMC content resulting in a thin vein wall where individual layers could not be distinguished. In hypertrophic segments an increase in ECM broke up the smooth muscle bundles. The intima was often thickened with hyperplasia and hypertrophy of the longitudinal SMC, consistent with an earlier report.[4]

Using smooth muscle actin and P2 receptor antibodies, double staining identified $P2X_1$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors on longitudinal and circular smooth muscle. There was no immunostaining for $P2X_{2-6}$ or $P2Y_{11}$ receptors. $P2X_1$ receptor staining intensity was decreased in varicose (Fig. 1b) compared to control veins (Fig. 1a). Conversely, $P2Y_1$ receptor staining intensity was increased in varicose (Fig. 1f) compared to control veins (Fig. 1e). $P2Y_2$ receptor staining (Fig. 2a and b) was weak in both tissue groups, but its intensity was increased on intimal longitudinal muscle in most varicose veins. $P2Y_4$ (Fig. 2e and f) and $P2Y_6$ (Fig. 3a and b) receptors stained with similar intensities in control and varicose sections.

Preabsorption of the primary antibody with its corresponding peptide eliminated immunofluorescence for all receptors in control and varicose vein (Fig. 1c,d,g,h, 2c,d,g,h, 3c and d).

The boxed areas in Fig. 1a and f are represented in Fig. 4 at increased magnification. The red $P2X_1$ staining (Fig. 4a) and the green smooth muscle actin staining (Fig. 4b) on the same section are shown. Colocalization of actin and $P2X_1$ receptors appear yellow (Fig. 4c) Similarly, $P2Y_1$ receptors (red; Fig. 4d) and actin (green; Fig. 4e) are colocalised (yellow; Fig. 4f).

Electron microscopy

Structures were identified in healthy (control) veins characteristic of a smooth muscle contractile phenotype, including numerous myofilaments that attach

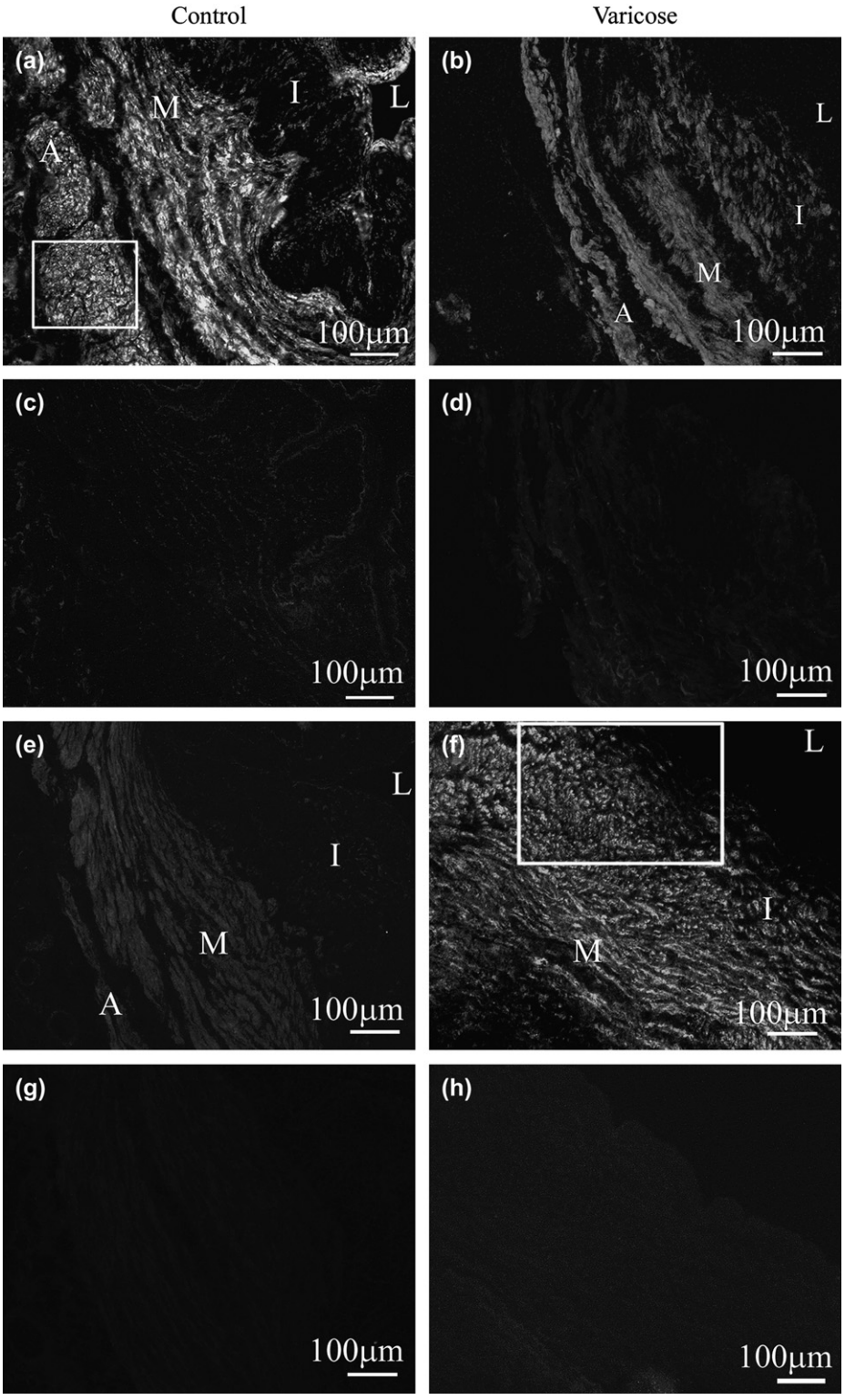

Fig. 1. Immunofluorescent staining of transverse sections of LSV. $P2X_1$ receptors on control (a) and varicose (b) veins, and $P2Y_1$ receptors on control (e) and varicose (f) veins. $P2X_1$ preabsorption control of control (c) and varicose (d) veins, $P2Y_1$ controls of control (g) and varicose (h) veins. (L = lumen, I = intima, M = media, A = adventitia). Boxed areas in (a) and (f) represent areas magnified in Fig. 4 (a–f, respectively).

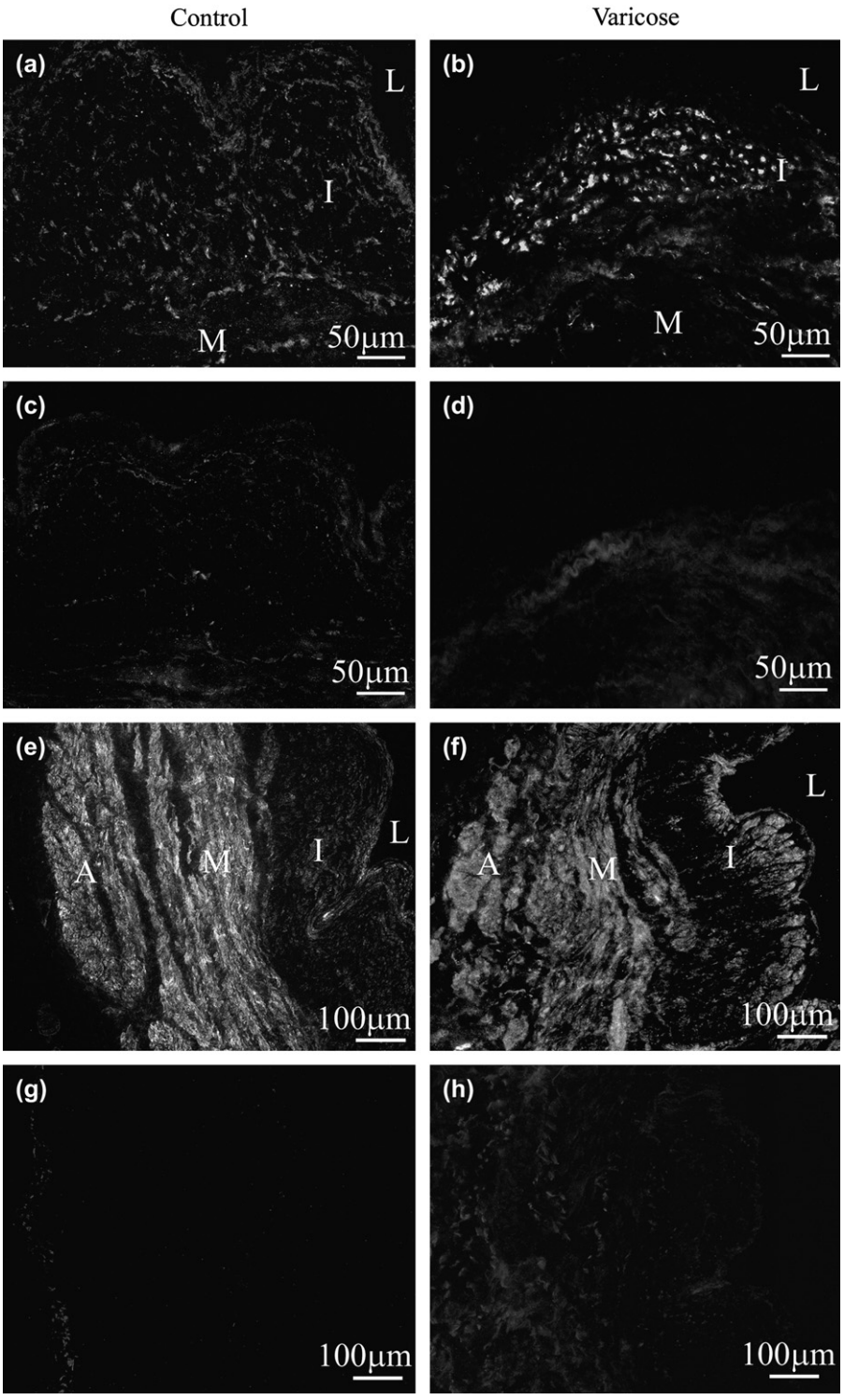

Fig. 2. Immunofluorescent staining of transverse sections of LSV. $P2Y_2$ receptors on control (a) and varicose (b) veins, and $P2Y_4$ receptors on control (e) and varicose (f) veins. $P2Y_2$ preabsorption control of control (c) and varicose (d) veins, and $P2Y_4$ controls for control (g) and varicose (h) veins. (L = lumen, I = intima, M = media, A = adventitia).

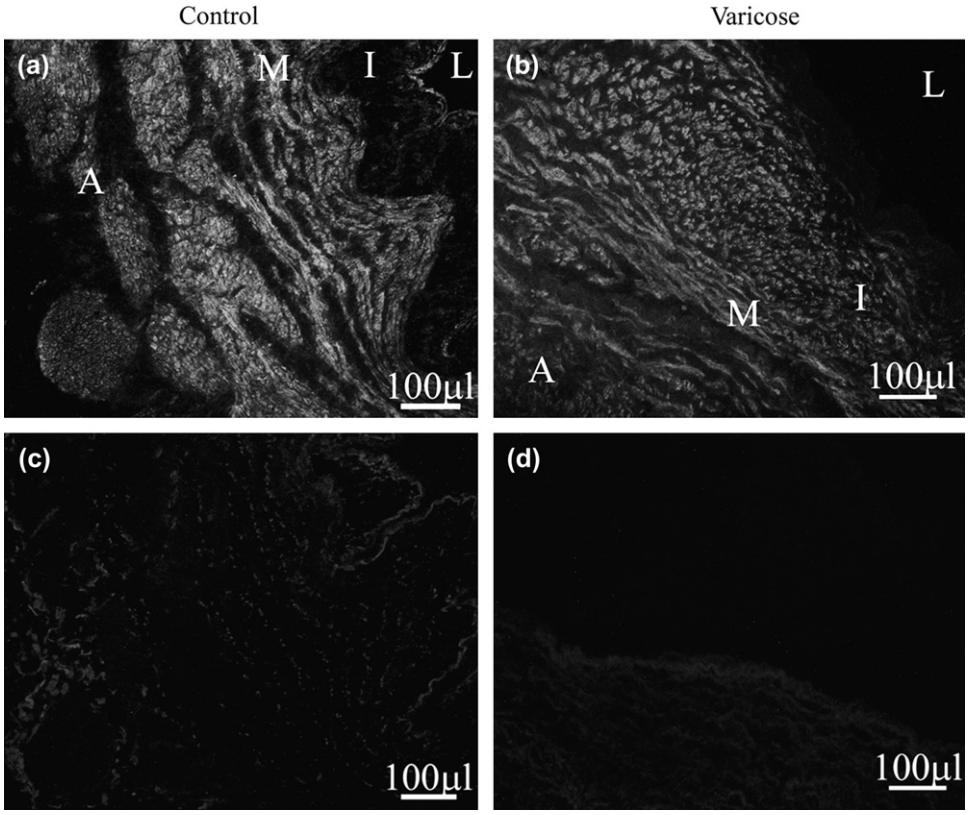

Fig. 3. Immunofluorescent staining of transverse sections of LSV. $P2Y_6$ receptors on control (a) and varicose (b) veins. $P2Y_6$ preabsorption controls for control (c) and varicose (d) veins. (L = lumen, I = intima, M = media, A = adventitia).

to dense bodies within the cytoplasm and in dense areas of the plasma membrane that alternate with caveolae. Organelles (including mitochondria and golgi complexes) are located in the perinuclear region (Fig. 5a). SMC were tightly packed in organised bundles and surrounded by fibrous tissue (Fig. 6a).

Varicose SMC had properties characteristic of a synthetic phenotype. Whilst some varicose SMC showed characteristics similar to control, other varicose SMC contained an increased volume of organelles (including vesicles and dilated rough endoplasmic reticulum) located at the periphery of the cell (Fig. 5b). These cells, though synthetic in appearance, were confirmed as being a SMC by the presence of a continuous basal lamina with caveolae and a limited appearance of myofilaments and dense bodies. The varicose SMC showing synthetic properties were located around the intimal and inner medial layers. Due to the disorganisation of muscle fibres seen in varicose veins, it was not possible to distinguish whether they were longitudinal or circular fibres. No synthetic phenotyped SMC was seen in the adventitia. Low magnification of the varicose sections revealed an increase in collagen and elastic tissue separating the muscle bundles, compared to control sections (Fig. 6b).

Functional experiments on circular smooth muscle

LSV circular muscle contracted to NA and KCl, and contractions were reduced in the varicose tissue ($P < 0.0001$ and $P = 0.0338$, respectively) (Fig. 7a). Circular muscle contracted to ATP ($P = 0.0178$) and α,β-meATP ($P = 0.0292$) (Fig. 7b), contractions were significantly reduced in the varicose tissue.

ACh failed to induce relaxation of NA (EC_{50} concentration) precontracted control or varicose vessels, suggesting that the endothelium was significantly disrupted (figure not shown).

Functional experiments on longitudinal smooth muscle

Longitudinal muscle in healthy and varicose vein contracted to purinoceptor and adrenoceptor agonists. There was a significant reduction in contractions of the varicose tissue to NA ($P < 0.0001$), KCl ($P = 0.0324$), ATP ($P < 0.0001$) and α,β-meATP ($P = 0.0033$) (Fig. 7c and d).

Unlike circular muscle, longitudinal muscle contracted to several P2Y receptor agonists. Significant reductions in contractions were again seen in varicose

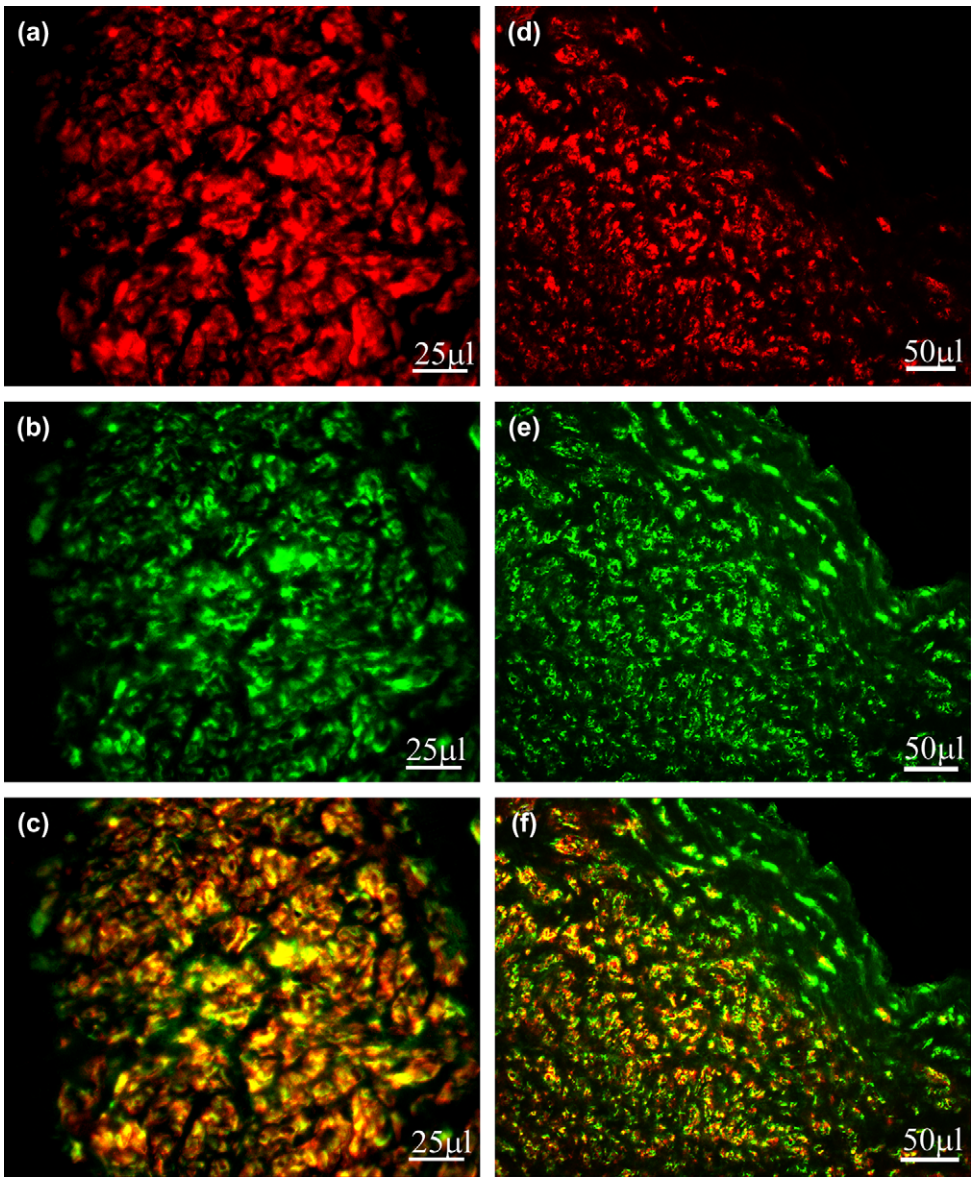

Fig. 4. Immunostaining of transverse sections of LSV, outlined by boxes in Fig. 1(a and f). $P2X_1$ receptors (red) in control vein (a) and $P2Y_1$ receptors (red) in varicose vein (d). Smooth muscle actin (green) (b, e). Colocalisation (yellow/orange) of $P2X_1$ receptors (c) and $P2Y_1$ receptors (f) and actin on SMC.

tissue to 2-MeSADP ($P = 0.0091$), UTP ($P = 0.0369$) and UDP ($P = 0.292$) (Fig. 8a–c) compared to control tissue.

In both control and varicose tissue, contractions to 2-MeSADP were significantly reduced ($P = 0.0001$ and $P = 0.0125$, respectively) in the presence of MRS2179 (10^{-6} M). Contractions to UDP were also significantly reduced in the presence of cibacron blue 3GA (10^{-4} M) (varicose tissue, $P < 0.0001$ and control tissue, $P = 0.0008$) (Fig. 8d and e). Contractions to UTP were inhibited by suramin (10^{-4} M) (control ($P = 0.0009$) and varicose ($P = 0.0023$). UTP is an agonist at both $P2Y_2$ and $P2Y_4$ receptors, however suramin distinguishes between suramin-sensitive $P2Y_2$ and suramin-insensitive $P2Y_4$ receptors.[9]

Electrical field stimulation induced frequency-dependent contractions of longitudinal muscle. Prazosin significantly reduced contractions ($P < 0.0001$) and PPADS reduced contractions even further ($P = 0.0481$). TTX demonstrated that responses were nerve-mediated (Fig. 8f).

Discussion

There was a significant age difference between the control and varicose vein groups, with more females

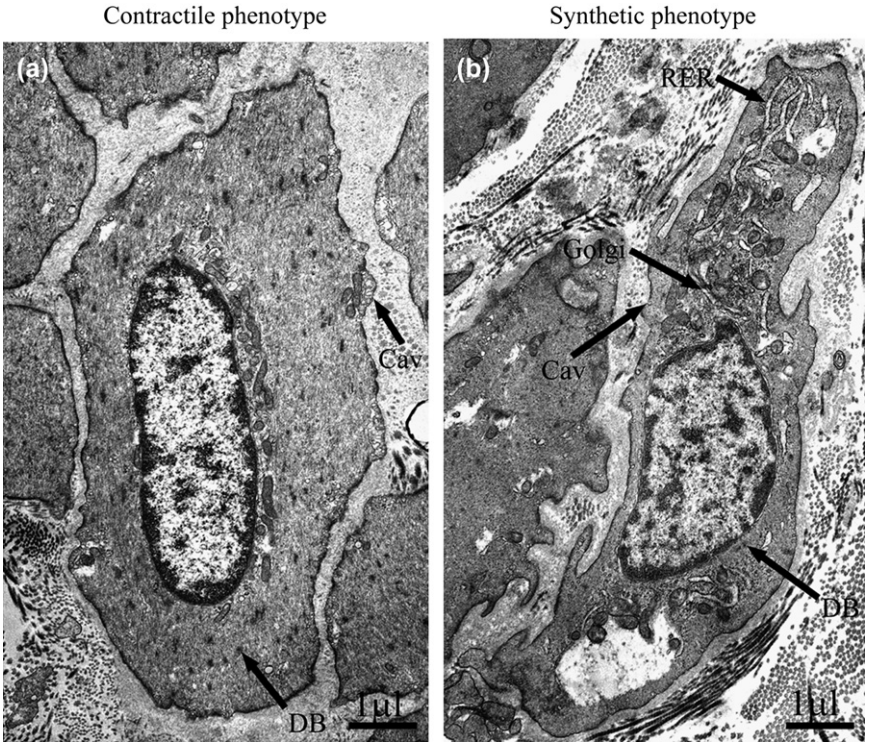

Fig. 5. Electron micrograph of SMC demonstrating the contractile phenotype in control veins (a) and the synthetic phenotype in varicose veins (b). Organelles appear perinuclear in the contractile phenotype, and towards the cell periphery in the synthetic phenotype. (Cav = caveolae, RER = rough endoplasmic reticulum, DB = dense bodies).

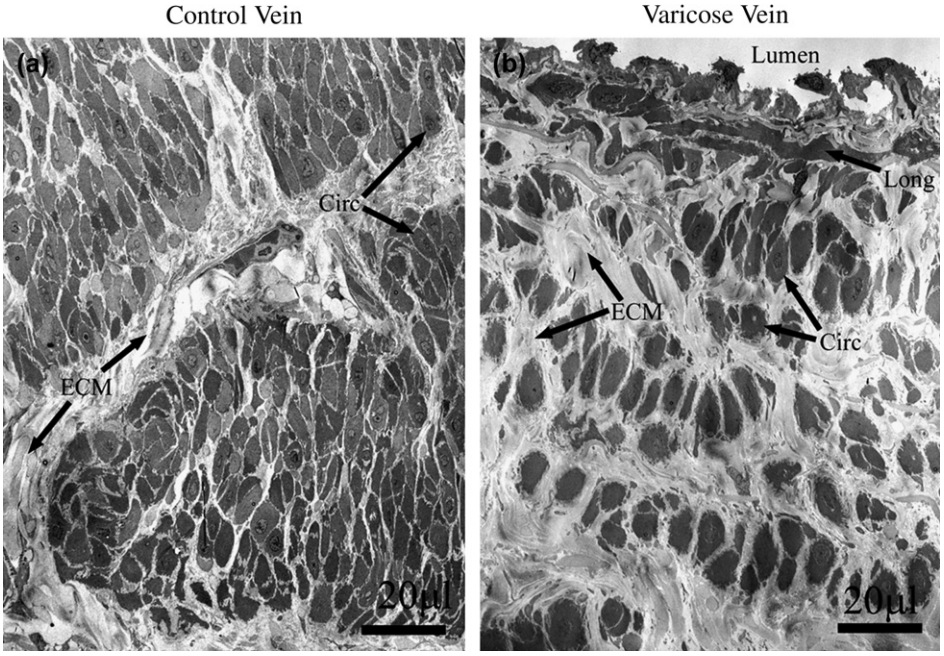

Fig. 6. Electron micrograph of longitudinal sections of LSV comparing the closely bound muscle bundles in control vein (a) with the SMC that are separated by an increase in the extracellular matrix in varicose vein (b). (ECM = extracellular matrix, Long = intimal longitudinal muscle, Circ = circular muscle).

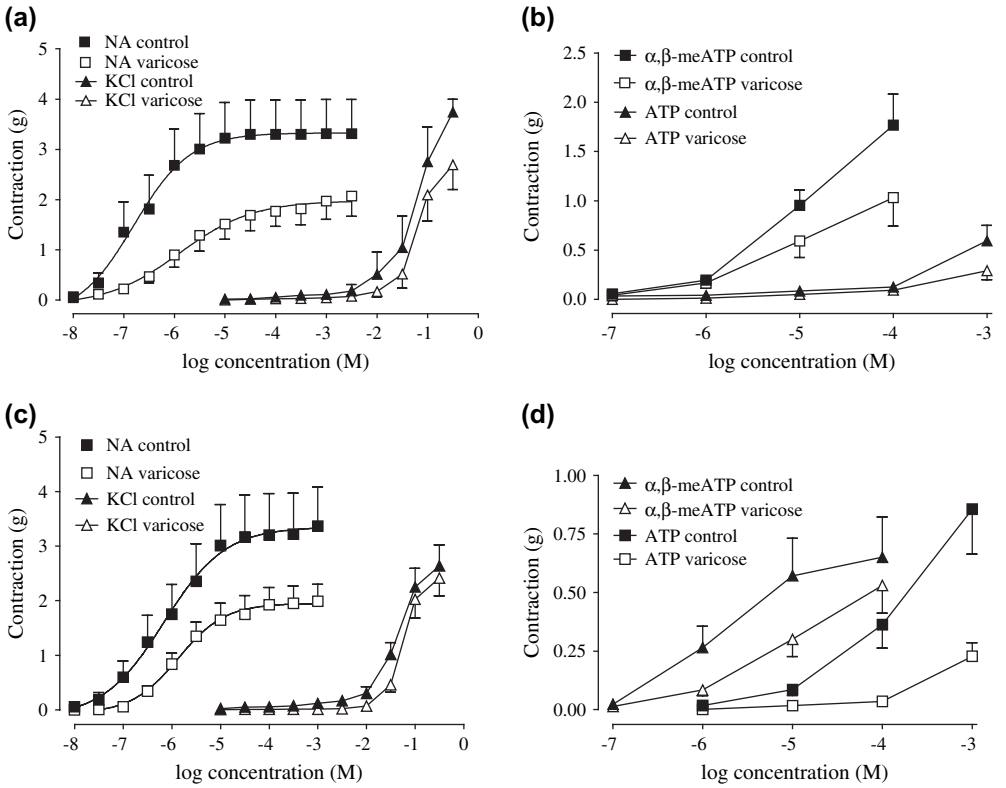

Fig. 7. Concentration-response curves of circular (a, b) and longitudinal (c, d) smooth muscle of control and varicose human long saphenous vein. NA (10^{-8} to 10^{-3} M) and KCl (10^{-5} to 3×10^{-1} M) contractions (a, c), and α,β-meATP (10^{-8} to 10^{-5} M) and ATP (10^{-6} to 10^{-3} M) contractions (b, d) are shown. All symbols represent mean ± SE (unless masked by the symbol).

in the varicose group as oestrogen is a causal factor.[18] During pregnancy, varicose vein presentation increases with plasma oestrogen levels, but usually resolves post partum. Contributing factors during pregnancy may include impaired venous drainage from the lower limbs. Oestrogen receptors have been identified on varicose and control LSV[19] and are thought to affect vascular wall strength by relaxing smooth muscle and softening collagen fibres. Haynes *et al*.[20] showed that oestrogen increases $P2X_1$ and $P2X_7$ receptor-mediated contractions in uterine arteries. UTP was more potent in oestradiol-treated animals by an upregulation of a UTP-specific pyrimidine receptor subtype.

Hypertrophy of the intimal longitudinal muscle layer in varicose veins is consistent with previous studies.[21] An increase in cell size with no changes in cell number, suggesting hypertrophy but not hyperplasia has been reported in hypertensive rat portal vein.[22] Theories for the intimal changes include hypoxia of the endothelial cells[23] and endothelium disruption causing SMC exposure to blood flow leading to modulation of its function.[24] This was suggested by Lee *et al*.[25] and supported by increases in SMC and ECM (collagen) in the LSV.[26] It has been proposed that varicose changes are due to modulation of their normal function.[21] The separation of the SMC by increased ECM in the hypertrophic intima suggests they have proliferative and synthetic functions and have adopted a different phenotype.[3] Aortocoronary saphenous vein grafts have intimal hyperplasia in response to mechanical injury and haemodynamic disturbances.[3] A similar physiological exposure occurs in the LSV due to increased venous pressure, perhaps promoting the SMC to change phenotype. Buján *et al*.[27] found a higher metabolism of the elastic component in varicose veins due to a restructuring of the elastic component of the vein wall, as a consequence of alterations in the transcription mechanisms of muscle cells.

The electron microscopy findings in control veins were characteristic of contractile SMC as previously reported.[17] Populations of poorly differentiated SMC with an increase in secretory cytoplasmic organelles and a reduction in filament bundles, characteristics seen in synthetic phenotyped SMC, have been previously noted in LSV, [17] in addition to phagocytic and secretory properties.[4,5] The ECM seen in varicose veins could be synthesised via synthetic phenotyped SMC.

In the varicose vein wall, a significant increase in transforming growth factor β1 (which stimulates the

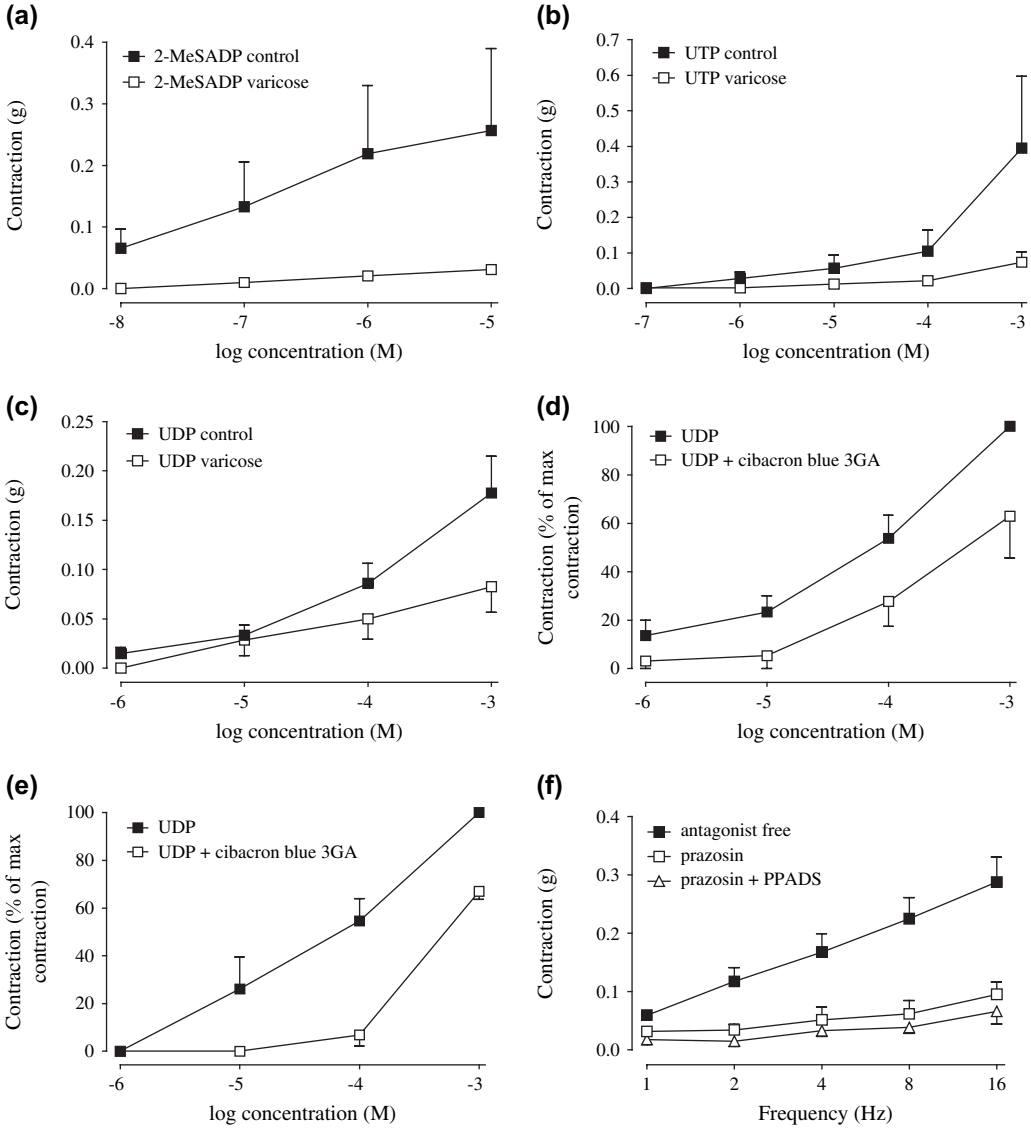

Fig. 8. Concentration response-curves of control and varicose longitudinal smooth muscle from human long saphenous vein to P2 receptor agonists, and in the absence and presence of P2 receptor antagonists. (a) 2-MeSADP (10^{-8} to 10^{-5} M); (b) UTP (10^{-6} to 10^{-3} M); (c) UDP (10^{-6} to 10^{-3} M). Concentration response curves to the $P2Y_6$ receptor agonist UDP (10^{-6} to 10^{-3} M) in the absence and presence of cibacron blue 3GA (10^{-4} M) in control (d) and varicose (e) LSV. (f) Electrical field stimulation of longitudinal smooth muscle in control LSV in the absence and presence of prazosin (10^{-6} M), then in the presence of prazosin and PPADS (3×10^{-5} M). All symbols represent mean ± SE (unless masked by symbol).

synthesis of ECM components, especially collagens and elastin, reduces the expression of matrix metalloproteinases and increases expression of tissue inhibitors) and an increase in the cytokine basic fibroblast growth factor (known to be chemotactic and mitogenic for SMC) has been reported, with no variation in vascular endothelial growth factor.[3] Inflammatory cells could not account for the cytokine modulation, as they were not present. These three mechanisms could increase the ECM in varicose veins.

Previous studies on LSV have shown reduced contractility of the circular muscle in the varicose state to vasoconstrictors.[6,28] $P2X_1$, $P2Y_2$, $P2Y_6$ receptors have been identified on the circular smooth muscle[14,15,28] but for the first time we show a reduction in contractility through $P2X_1$ receptors in the varicose tissue as well as to NA and KCl, which is consistent with previous studies.[6,28] Reduced contractions may result from a combination of decreased muscle volume and weaker contractile muscle cells in the varicose vein.

For the first time contractions of the longitudinal smooth muscle of human LSV was demonstrated and shown to be mediated by $P2X_1$, $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors. mRNA for $P2Y_1$ receptors has been detected on endothelium[29] and endothelium-denuded healthy LSV,[30] but no contractile property has been demonstrated. Endothelial $P2Y_1$ receptors usually mediate vasodilatation, however, $P2Y_1$ receptors here mediate contraction of the longitudinal muscle, albeit reduced in varicose tissue. Contractile properties of longitudinal muscle has been previously reported in the human coronary artery and rat portal vein.[31,32]

SMC and endothelial cell proliferation, death and secretory properties are important in new vessel growth, during wound healing and with intimal thickening during arterial diseases.[33,34] Purinoceptors play important roles in the signalling pathways of these events.[8,35] Vasoconstriction in blood vessels is mediated mainly by $P2X_1$ receptors and by $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors,[7,36] while mitogenic effects of SMC are mediated by $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors.[7,35] Transition from contractile to synthetic SMC phenotypes has been shown in atherosclerosis, restenosis after angioplasty and during cell culture *in vitro*.[35,37] mRNA levels of $P2Y_1$ and $P2Y_2$ receptors increase 342-fold and 8-fold, respectively, in cultured rat aortic smooth muscle cells that show the synthetic phenotype when compared to phenotypic contractile cells in freshly dissociated muscle.[38] $P2X_1$ receptor mRNA was detected in contractile cells but not in synthetic cells. mRNA levels of $P2Y_4$ and $P2Y_6$ receptors were similar in both cell phenotypes. $P2Y_4$ and $P2Y_6$ receptor activation stimulates mitogenesis in SMC.[39,40] The SMC in varicose LSV show similar changes in expression, i.e. reduced $P2X_1$ receptor immunostaining and $P2X_1$-mediated contraction $P2Y_1$ and $P2Y_2$ receptor immunostaining intensity increased, associated with increased synthetic and proliferative activity, and loss of contractile activity. Immunostaining intensity remained constant for $P2Y_4$ and $P2Y_6$ receptors, although there was a reduction in $P2Y_6$ receptor-mediated contractions in varicose veins.

Endothelial cell production of nitric oxide and increased sensitivity of the smooth muscle is thought to lead to increased synthesis of cyclic GMP and heightened vascular relaxation.[41] A mitogenic phenotype may account for this altered sensitivity. In aortic SMC, A_{2B} receptor activation inhibits growth,[42] and either $P2Y_2$ or $P2Y_4$ receptor activation stimulates cell proliferation.[43] In atherosclerosis, there is an upregulation of $P2Y_2$ receptors by a mitogen-activated protein kinase (MAPK)-dependent growth factor. Suramin inhibits platelet derived growth factor (PDGF) receptor activation and signalling through the MAPK-activator protein 1 pathway. PDGF is a growth factor antagonist inhibiting cell proliferation and reducing neointimal thickness in LSV grafts in mice.[44] These findings suggest an increased $P2Y_2$ receptor expression in a hypertrophied intima, similar to our demonstration of increased $P2Y_2$ receptor expression in varicose intima.

Intimal longitudinal smooth muscle is more prominent in hypertrophied varicose vein segments as described in this study. We propose that intimal longitudinal smooth muscle undergoes a change from contractile to synthetic phenotype in varicose veins. This is supported by the increase in intimal smooth muscle volume, a reduction in its contractile strength, increases in intimal ECM, increased intensity of intimal $P2Y_1$ and $P2Y_2$ receptor staining and reduced intimal $P2X_1$ receptor staining.

What is the source of the ATP that acts on the P2 receptors expressed on the smooth muscle of LSV? ATP may be released as a cotransmitter with NA from perivascular sympathetic nerves,[45] or released from endothelial cells during changes in flow (shear stress) and hypoxia.[46] A further question that will need to be resolved is whether the changes in smooth muscle phenotype and associated changes in purinergic signalling are causal or consequential in varicose vein development.

An understanding of these changes occurring within the purinergic signalling pathway may identify targets for therapeutic intervention. Modulation of vein muscle phenotype may be a useful approach for treating varicose veins and subsequent chronic venous insufficiency. It could potentially optimise the role of the long saphenous vein as a bypass graft.

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