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ORIGINAL PAPER

Purinoceptor expression on keratinocytes reflects their function on the epidermis during chronic venous insufficiency

Matthew J. Metcalfe · Daryll M. Baker · Geoffrey Burnstock

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Abstract Purines are extracellular nucleotides that have long-term effects on keratinocyte proliferation, differentiation and death through $P2Y_1$, $P2Y_2$, $P2X_5$ and $P2X_7$ receptors. This study examined changes in expression of these P2 receptors on lower leg epidermal keratinocytes in control and chronic venous insufficiency (CVI) states. Lower limb skin biopsies from CVI (CEAP classification 4a and 4b) and control skin were immunostained for the above P2 receptor subtypes and epidermal area was calculated. Our results with CVI show an increase in $P2Y_1$ and $P2Y_2$ receptor expression in basal and spinosal layers of the epidermis and an increase of $P2X_5$ receptors mainly in the spinosal layer and extending further into the stratum granulosum. In contrast, $P2X_7$ receptors were reduced in the stratum corneum in CVI. In conclusion, a thinner epidermis was found in CVI, which might be the result of the changes in expression of P2Y and P2X receptors on keratinocytes: that is, increased proliferation via $P2Y_1$ and $P2Y_2$ receptors and reduced $P2X_7$ receptor-mediated cell death opposed by a dominant decrease in cell numbers as a result of increased $P2X_5$ receptor-mediated differentiation (which is in effect antiproliferative). Thus, increased keratinocyte $P2X_5$ receptor activity may, in part, be accountable for epidermal thinning in CVI.

M. J. Metcalfe · G. Burnstock (✉)
Autonomic Neuroscience Centre,
Royal Free and University College Medical School,
Rowland Hill Street, London, NW3 2PF, UK
e-mail: g.burnstock@ucl.ac.uk

D. M. Baker
Department of Vascular Surgery, Royal Free Hospital,
Pond Street, London, NW3 2QG, UK

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Introduction

Purines and pyrimidines are involved in a wide range of activities, including neurotransmission and neuromodulation [6]. They act on P2 receptors belonging to two families: P2X ligand gated ion channels and P2Y G protein-coupled receptors [27]. Both short-term purinergic control of blood vessel tone and long-term roles of purines on vascular cell migration, proliferation, differentiation and death have been described [5, 7]. $P2Y_1$ and $P2Y_2$ receptors, identified in skin epidermis, are involved in keratinocyte proliferation, while $P2X_5$ receptors are associated with keratinocyte differentiation and $P2X_7$ receptors with keratinocyte cell death [14, 15].

Chronic venous insufficiency (CVI) is a functional disorder of the venous system of the lower limb, with venous hypertension the result of valve insufficiency. Additionally, venous outflow may be impaired due to obstruction [30]. Venous hypertension affects the overlying skin causing chronic inflammation and leads to varicose eczema, lipodermatosclerosis and ultimately venous ulceration. Different theories about the pathophysiological basis of these features exist. One is that increased venous pressure reduces perfusion pressure, resulting in white blood cells (WBCs) plugging the capillaries. The WBCs marginate and become activated [29], releasing enzymes and oxygen-free radicals, damaging surrounding tissues. Another theory is based upon the formation of a fibrin cuff. Raised venous pressure is thought to elongate the capillaries [31] and

widen pores between endothelial cells [22]. This allows larger molecules including fibrinogen to extravasate and accumulate. Upon conversion to fibrin this forms a barrier along with fibronectin and denatured collagen macromolecules [16], blocking the passage of nutrients and oxygen, leading to ischaemia and cell death. Both a perivascular leucocyte infiltration and the deposition of fibrin forming a fibrin cuff reflect an ongoing inflammatory process targeting the superficial layers of the skin leading to CVI changes [25].

The epidermis is a multilayered organ. It consists of rows of squamous epithelial cells that divide as they move from their basal layer, through the stratum spinosum, to the stratum granulosum, where they eventually flatten into cross linked keratin filaments forming the outermost layer, the stratum corneum. The roles of $P2X_5$, $P2X_7$, $P2Y_1$ and $P2Y_2$ receptors in these different layers in human skin have been described previously [14]. The balance between proliferation, differentiation and apoptosis of keratinocytes helps maintain an epidermis of constant thickness.

Our study was to examine the expression of $P2X_5$, $P2X_7$, $P2Y_1$ and $P2Y_2$ receptors in the epidermis of human lower leg skin and the changes seen in patients with CVI, which can result in lipodermatosclerosis and venous ulceration.

We examined skin samples from CVI patients undergoing stripping of their primary varicose veins. Reflux had been confirmed by either hand held doppler or venous duplex scanning by the vascular team prior to surgery. Skin biopsies at sites of CVI were taken from the medial aspect of the mid lower calf at the site of either the distal incision for stripping of the long saphenous vein (LSV) or at the site of a stab incision for avulsing a prominent varicose vein. Biopsies were not taken from the ankle. Elliptical skin biopsies were obtained from five patients (two males and three females) aged 46–69 years (mean = 56.8 years). Sites of CVI consisted of skin pigmentation in two patients (CEAP 4a) and lipodermatosclerosis in three patients (CEAP 4b), based on the clinical–etiology–anatomy–pathophysiology [1] classification. There were no coexisting medical conditions in these patients and none took any regular medications. Healthy control skin was obtained from five patients (three males and two females) aged 56–68 years (mean = 64.4 years), undergoing coronary artery bypass surgery involving harvesting of the LSV. Elliptical skin samples were excised from an edge of the LSV incision from the medial aspect of the mid lower calf, corresponding to the area CVI skin samples were obtained from. Control skin showed no signs of CVI and reflux was excluded by hand held doppler. Patients in the control group were taking the following cardiac medications for ischaemic heart disease including β blockers, diuretics, nitrates, ACE inhibitors and statins. Diabetic patients and patients with skin conditions (e.g. psoriasis, patients on steroids) were excluded from the study. Ethics approval was obtained by the joint UCL/ULCH Ethics Committees on Human Research and by the Royal Free Hampstead Research Ethics Committee.

Skin samples were collected in Hanks balanced salt solution (HBSS; Invitrogen Ltd, Paisley, UK) and frozen in isopentane, precooled in liquid nitrogen. Samples were sectioned at 10 μm on a cryostat (Reichert Jung CM1800), collected on gelatine-coated slides and air dried at room temperature. Slides were stored at −20°C.

Polyclonal $P2X_5$ and $P2X_7$ receptor antibodies (provided by Roche Palo Alto, CA, USA) and polyclonal anti-$P2Y_1$ and anti-$P2Y_2$ receptor antibodies (Alomone Laboratories, Jerusalem, Israel) were kept at −20°C.

Sections were fixed for 4 min in 4% formaldehyde in 0.1 M phosphate buffer solution (PBS) containing 0.2% picric acid, then washed with PBS. Sections were primarily blocked for 60 min in 10% normal horse serum (NHS) in 0.1 M phosphate buffer, containing 0.05% merthiolate. Sections were then incubated overnight with the primary receptor antibody at concentrations of 1:100–1:200 in 10% NHS in PBS with 0.05% merthiolate. On the second day, sections were washed in PBS and stained with the secondary antibody donkey antirabbit Cy3 (Jackson ImmunoResearch Laboratory, West Grove, PA, USA) at 1:300 in PBS–merthiolate for 60 min. Sections were washed, and mounted in Citifluor (Citifluor Ltd, London, UK).

Control experiments were performed by separately omitting the primary and secondary antibodies, and by preabsorbing the primary antibody with its corresponding peptide. Preabsorption was carried out by adding the peptide at a ratio of 1:1 in 10% NHS in PBS with 0.05% merthiolate, leaving for 12 h at 4°C, passing through a syringe filter (4 mm with a 0.2 μm PPmembrane) then centrifuged at 13,000 rpm for 5 min using only the supernatant.

Semi-quantitative assessments of changes in immunofluorescent intensity were performed blind by an independent observer.

H&E slides were prepared by fixing sections (4% paraformaldehyde, 10 min) prior to staining (20 min) with Ehrlichs haematoxylin. Following dipping in acid alcohol and washing (15 min), sections were then stained in eosin (5 min), before finally washing in water, 70% alcohol (1 min), 100% alcohol (6 min) and xylene (8 min). Sections were mounted in Eukitt.

Slides were photographed using a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) mounted

with a Leica DC 200 digital camera (Leica, Heerbrugg, Switzerland). Images were converted from colour to greyscale using Photoshop (Adobe 5.0, San Jose, USA).

Low magnification images of H&E stained epidermis were taken. Two sections from different areas of each skin sample from all five patients in each group were studied. The epidermal area was then calculated using a Scion Image programme and expressed as mean area (μm^2) ± standard error (n). Statistical analysis was carried out using an unpaired Student's t test, $P < 0.05$ was taken as significant.

Sections were cut sequentially. Low power magnifications of H&E stains of skin epidermis are shown (Figs. 1a, d, g, j and 2a, d, g, j).

Control skin stained for $P2Y_1$ (Fig. 1b) and $P2Y_2$ receptors (Fig. 1h) in the basal layer of the epidermis, with $P2Y_2$ receptors also present in the stratum spinosum (SS). In CVI skin, $P2Y_1$ receptor staining was markedly increased in the basal layer and present in the lower layers of the SS in four of the five patients (Fig. 1e), while $P2Y_2$ receptors were markedly increased in both stratum basale and spinosum, and extended further into the spinosal layer in all patients (Fig. 1k).

In control skin, $P2X_5$ receptors stained throughout the SS, but also in the basal and granulosal layers (Fig. 2b). In CVI skin $P2X_5$ receptor staining extended further into the stratum granulosum and immunostaining intensity was increased in all layers from all patients (Fig. 2e).

In control skin $P2X_7$ receptor staining was in the uppermost layer of dead cells, the stratum corneum (Fig. 2h). In CVI skin, $P2X_7$ staining was markedly reduced (Fig. 2k).

Control experiments (preabsorption with the corresponding peptide) for each P2 receptor antibody resulted in no specific immunostaining (Figs. 1c, f, i, l and 2c, f); for the $P2X_7$ receptor, some non-specific staining was observed (Fig. 2i, l). Due to the limited sample size, comparisons between CEAP 4a and 4b samples in the CVI group were not made.

The mean epidermal areas were calculated for two sections per skin sample from the control and CVI groups. The mean epidermal areas (μm^2) for control and CVI skin was 295 ± 6.7 (5) and 275 ± 5.4 (5), respectively (Fig. 3). The epidermal area in CVI skin was significantly ($P = 0.0313$) reduced, indicating that the epidermis was thinner.

Previous research on CVI skin has focused on changes occurring at the dermal capillaries. These pathophysiological changes may explain the skin changes seen in clinical practice. However, few studies have focused on the epidermis itself. White cell infiltration [28], fibrin deposition [16] and oedema all occur within the dermis leading to a reduced delivery of nutrient and oxygen [18] to the epidermis and an accumulation of waste products from a reduced blood flow away from the site affected. Oxidative stress and white cell extravasation releases inflammatory mediators [8], which are thought to damage the epidermis [32]. It is thought that ischaemia followed by reperfusion worsens the process, leading to the chronic inflammatory state [19]. Whilst dermal changes in CVI have been previously reported, little work exists to demonstrate the proliferative changes the keratinocytes undergo.

$P2Y_1$ and $P2Y_2$ receptors are known to mediate keratinocyte proliferation in the human epidermis and our controls match the findings of an earlier study of human 'leg' skin, although proliferation marker staining was not repeated [14]. The increased expression of $P2Y_1$ and $P2Y_2$ receptors on the basal layer and stratum spinosum in CVI skin suggests increased proliferation. The extension of $P2Y_2$ receptor staining towards the stratum granulosum suggests an increased keratinocyte proliferation extending beyond the basal layer deep into the epidermis. CK 14 is a cytokeratin found on basal cells that is lost upon keratinocyte differentiation. It is greatly increased in suprabasal layers in venous eczema and lipodermatosclerosis [26] suggesting prolonged proliferation of the basal cells. This is consistent with our findings of increased $P2Y_1$ and $P2Y_2$ receptors in layers beyond the stratum basale in CVI. Increased keratinocyte proliferation may act as a compensatory mechanism to maintain epidermal thickness in the presence of increased differentiation, preventing epidermal thinning and ulceration.

ATP and UTP released as part of an inflammatory response promote keratinocyte proliferation and inhibit differentiation through activation of the $P2Y_2$ receptors [11]. Antagonists to $P2Y_2$ receptors are therapeutic targets in keratinocyte hyperproliferation states such as psoriasis [11]. Hyperproliferative keratinocytes, demonstrated by an increase in integrin β1 [13], at the edge of venous ulcers is consistent with increased proliferation. The inflammatory process present in CVI may increase keratinocyte proliferation through increased activity of $P2Y_2$ receptors, accounting for their increased expression.

$P2X_5$ receptors are restricted to metabolically active, differentiating cell layers of the epithelia and are not associated with mitosis and cell death [15]. $P2X_5$ receptors have been detected in spinous and granular layers, decreasing in its intensity towards the outermost layer [15]. Greig et al. [14] showed the presence of $P2X_5$ receptors in these three layers in human 'leg' skin, with early keratinocyte differentiation

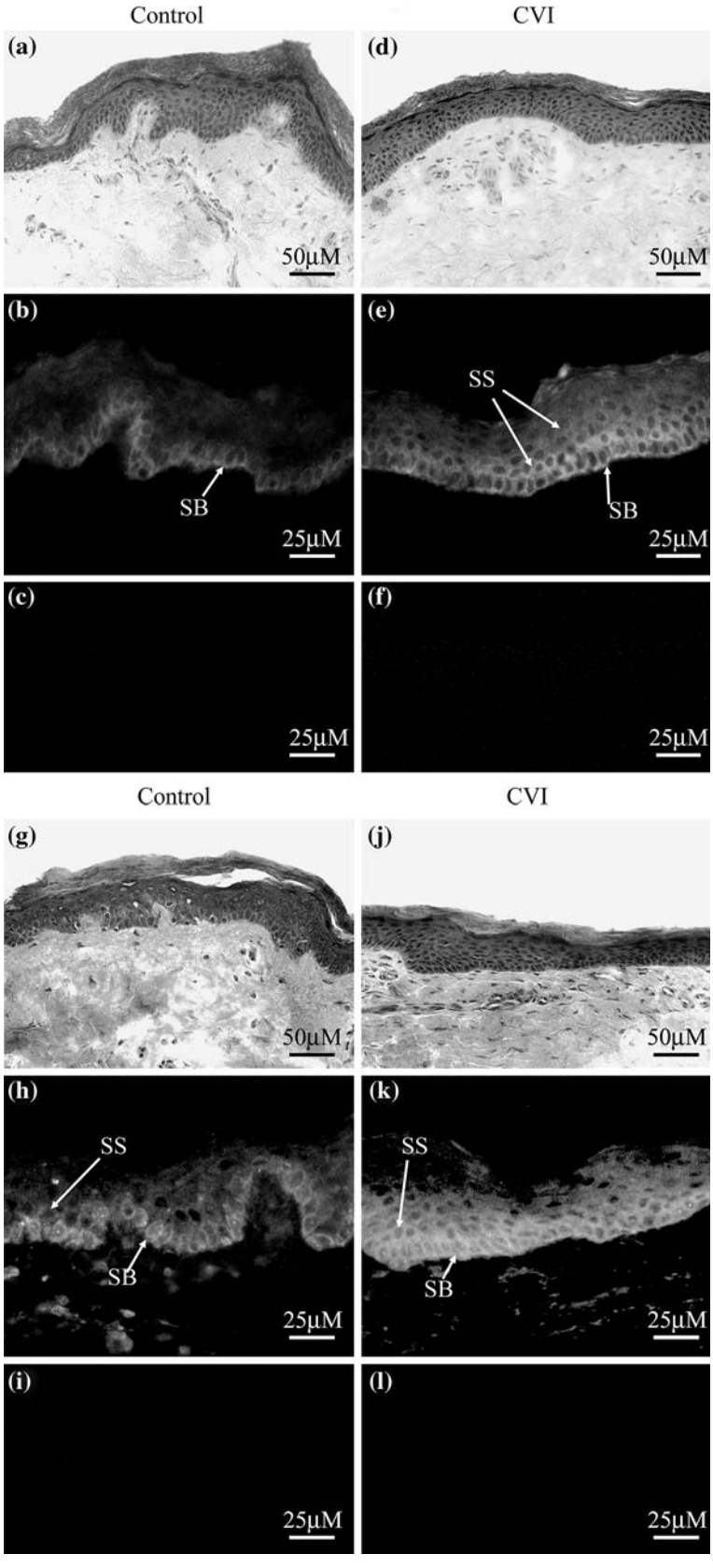

Fig. 1 H&E staining and $P2Y_1$ and $P2Y_2$ receptor immunostaining of human epidermal keratinocytes. H&E staining of controls **a** and **g** and CVI **d** and **j** skin sections. At higher magnification, immunofluorescence staining on stratum basale (*SB*) in the control **b**, with increased staining in basal and spinosal (*SS*) layers in CVI **e**. Preabsorption of $P2Y_1$ receptors with its peptide shows minimal immunofluorescence **c** and **f**. Immunofluorescence staining of $P2Y_2$ receptors is present on stratum basale (*SB*) and lower stratum spinosal (*SS*) layers in control skin **h**. Immunostaining extends deeper into the spinosal (*SS*) layer and is of a greater intensity in CVI **k**. Preabsorption of $P2Y_2$ receptors with its peptide shows minimal immunofluorescence **i** and **l**

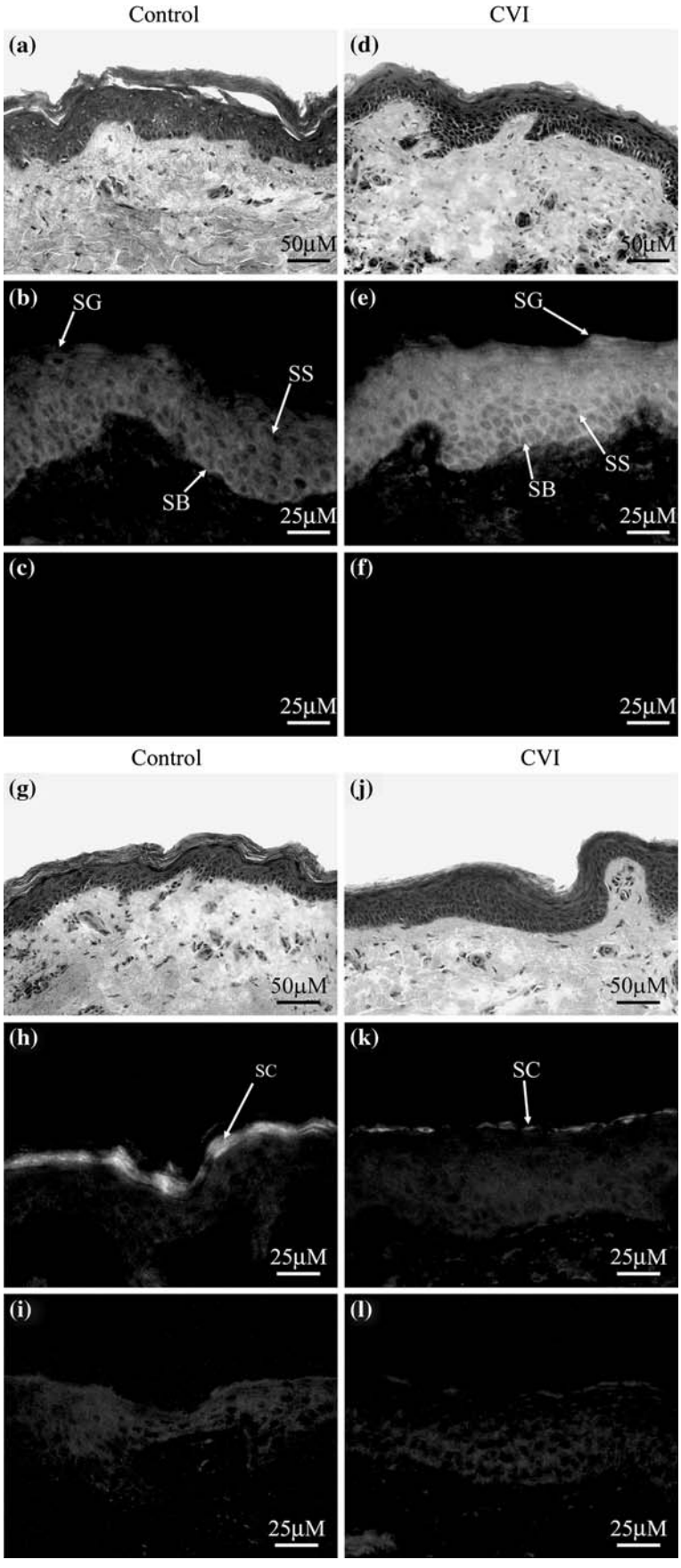

Fig. 2 H&E staining and $P2X_5$ and $P2X_7$ receptor immunostaining of human epidermal keratinocytes. H&E staining of controls **a** and **g** and CVI **d** and **j** skin sections. At higher magnification, immunofluorescence staining of $P2X_5$ receptors is present on basale (*SB*), spinosal (*SS*) and stratum granulosum (*SG*) layers in control skin **b**. Skin in CVI shows the same pattern of staining but of increased intensity **e**. Preabsorption of $P2X_5$ receptors with its peptide shows minimal immunofluorescence **c** and **f**. Immunofluorescence staining of $P2X_7$ receptors is present on the stratum corneum (*SC*) in control skin **h**, but staining is markedly reduced in CVI **k**. Preabsorption of $P2X_7$ receptors with its peptide shows reduced immunofluorescence **i** and **l**

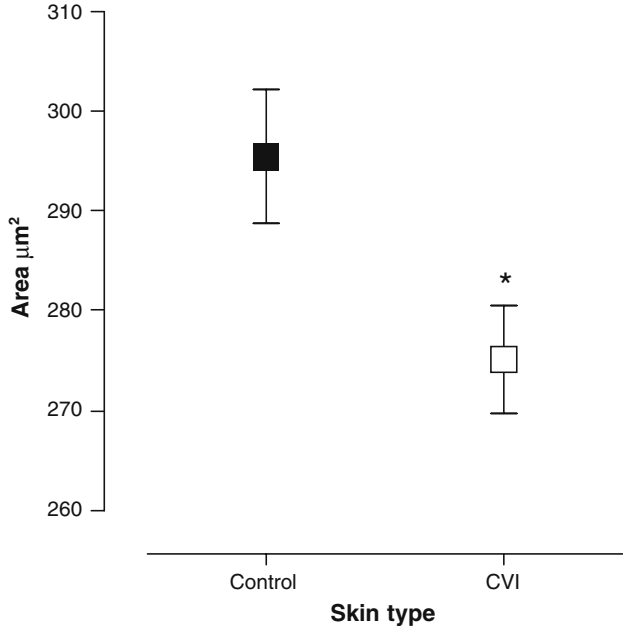

Fig. 3 Bar chart representing epidermal area in low magnification images of control and CVI skin. Mean epidermal area (μm^2) ± standard error ($n = 5$) of control (*black square*) and CVI (*white square*) skin are shown. The mean of the CVI skin is significantly thinner than control epidermis (*$P = 0.0313$)

occurring in the stratum spinosum, and late differentiation occurring within the upper spinosal and granular layers. These findings match our control $P2X_5$ receptor staining. In CVI skin, the intensity of $P2X_5$ receptor staining increased throughout all three layers suggesting an overall increase in $P2X_5$ receptor-mediated keratinocyte differentiation, which in effect is antiproliferative. Some cytokeratins expressed on epithelial cells are established markers of differentiation. CK 10, a marker of terminal differentiation, has been shown to increase in venous eczema and lipodermatosclerosis in suprabasal layers [26]. This is consistent with our findings of increased $P2X_5$ receptor expression representing an increase in differentiation in spinosal and granular layers, although staining with differentiation markers was not repeated.

$P2X_7$ receptors on macrophages and lymphocytes have cytotoxic functions at sites of inflammation [20], possibly involved in interleukin-1β (IL-1β) release [3], mitogenic stimulation of T lymphocytes [2], and cytoplasmic communication between macrophages and lymphocytes [9]. $P2X_7$ receptor levels on monocyte–macrophage lineage cells are increased in sarcoidosis, where they are associated with cytotoxicity, maturation and IL-1β release [24]. $P2X_7$ receptor staining was seen on the outer layer of dead keratinocytes, the stratum corneum, on control skin [15], and represents keratinocytes terminally differentiating [14]. In CVI, $P2X_7$ receptor staining was markedly reduced to small, scattered areas of the stratum corneum. $P2X_7$ receptors are involved in the induction of cell death [12, 33], and might be a compensatory change, which prevents further thinning of the epidermis.

Plasma levels of ICAM-1 (intercellular adhesion molecule-1) are increased in response to venous hypertension [29]. Increased expression of ICAM-1 in the capillaries and T-lymphocytes and macrophage infiltration around vessels are seen in patients with lipodermatosclerosis [8]. Allopurinol, a xanthine oxide inhibitor, downregulates the expression of ICAM-1 and $P2X_7$ receptors on macrophages [23]. ICAM-1 on macrophages attaches to T lymphocytes allowing antigen presentation and T cell activation. Thus, allopurinol may suppress T cell activation and may suppress keratinocyte $P2X_7$ expression and reduce keratinocyte cell death. Epidermal mast cells are found in chronic skin inflammation with hyperproliferative epidermis and in chronic ulcers, where mast cell granules are found inside keratinocytes. The mast cell mediators histamine and heparin, and human mast cell lysate have an inhibitory effect on keratinocyte proliferation and epithelial growth. It could be concluded that mast cells have an inhibitory effect on epidermal growth [17]. ATP induced histamine release from mast cells is mediated via $P2X_7$ receptors [10, 21]. Inhibition of keratinocyte growth may be a result of the action of ATP on mast cells. Mast cells may also act directly on keratinocyte P2 receptors by releasing ATP and affecting keratinocyte growth [4].

Our results show that CVI skin is thinner than controls. If the thickness of skin decreases with CVI then over time this would lead to continuous thinning of the epidermis until it eventually breaks down, ulcerating. This would follow the clinical picture. In our study, we have looked at preulcerated states. Studies have shown that impaired epithelialisation of chronic ulcers is not caused by the lack of epidermal stem cells, inadequate proliferation, differentiation or apoptosis at the edge of wounds [13]. Failure of wound healing may reflect the distorted organisation of the wound bed caused by infection and impaired nutrient supply, altering keratinocyte migration at the ulcer edge [13].

In summary, increased expression of $P2Y_1$, $P2Y_2$ and $P2X_5$ receptors and decreased expression of $P2X_7$ receptors has been shown in CVI. Whether the thinner epidermis in CVI might be the result of increased keratinocyte proliferation via $P2Y_1$ and $P2Y_2$ receptors, reduced apoptosis via $P2X_7$ receptors and a dominant antiproliferative effect mediated via $P2X_5$ receptors, requires further investigation. Enhanced keratinocyte proliferation through targeting $P2Y_1$ and

$P2Y_2$ receptors may increase epidermal thickening, protecting against breakdown and ulceration. Antagonising the actions mediated by $P2X_5$ receptors may reduce its antiproliferative effects resulting in a thicker epidermis. It is not clear whether these changes in purinoceptor expression found in CVI are of compensatory advantage or a secondary consequence.

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