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# Immunolocalization of protease-activated receptor-2 in skin: receptor activation stimulates interleukin-8 secretion by keratinocytes *in vitro*

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## SUMMARY

The protease-activated receptor-2 (PAR-2) is a seven transmembrane domain receptor related to the thrombin receptor, which is activated *in vitro* by cleavage by trypsin. Affinity-purified rabbit IgG raised against a peptide corresponding to the trypsin cleavage site of PAR-2 was used for an immunohistochemical study of skin. The expression of PAR-2 in epidermis was striking, with keratinocytes showing abundant intercellular and cytoplasmic staining. Basal cells showed the strongest staining intensity and the stratum corneum was negative. Staining with control IgG used at the same concentration was consistently negative. The functional expression of PAR-2 by the simian virus transformed human skin keratinocyte cell line SVK14 was demonstrated by Northern blot analysis, flow cytometric analysis and the measurement of intracellular calcium. Treatment of SVK14 with trypsin or a receptor agonist peptide (SLIGKV-$NH_2$) caused a dose-dependent increase in the secretion of the chemokine interleukin-8 (IL-8) *in vitro*. The effect of the peptide was specific, since control acetylated peptide was without activity. We conclude that PAR-2 is highly expressed by epidermal keratinocytes and receptor activation *in vitro* leads to increased IL-8 secretion by keratinocytes. These data raise the possibility that PAR-2 may play a role in epidermal homeostasis and inflammatory conditions.

## INTRODUCTION

A new family of cell surface receptors is emerging, in which specific protease ligands act as cytokines.[1] Protease-activated receptors (PAR) are activated by enzyme binding and cleaving the extracellular domain to create a new N terminus which acts as a tethered ligand.[2] The receptor may also be activated by short synthetic peptides based on the amino acid sequence of the tethered ligand N terminus revealed after cleavage.[3]

The first receptor of this type to be cloned was the human thrombin receptor, now designated PAR-1, and many of the cellular effects of thrombin appear to be mediated by this receptor. However a second thrombin receptor, termed PAR-3, has recently been identified, the distribution and function of which have yet to be fully established.[4]

The protease-activated receptor-2 (PAR-2) was isolated by low-stringency screening a genomic library for G-protein-coupled receptors.[5] Like PAR-1, it is activated by proteolytic cleavage or a peptide sequence corresponding to the new N terminus created by proteolysis. PAR-2 is expressed by vascular endothelial cells[6] and keratinocytes[7] but the distribution and function of PAR-2 expressed by keratinocytes in skin is unknown. There is increasing awareness that PAR-2 may play a role in acute inflammation. For example, neutrophils express PAR-2 and receptor activation rapidly stimulates an intracellular calcium flux and the increased expression of cell activation adhesion molecule CD11b.[8,9] Also, PAR-2 mRNA and protein expression levels increase 5–10-fold in vascular endothelial cells *in vitro* after treatment with the proinflammatory cytokines interleukin-1 (IL-1) and tumour necrosis factor on a similar time course of induction as intercellular adhesion molecule-1 and vascular cell adhesion molecule-1.[6]

Inflammatory skin conditions such as psoriasis are characterized by increased proteolytic activity in the epidermis,[10,11] the accumulation of neutrophils,[12] and thus the excessive production of the neutrophil chemokine IL-8 has been implicated in the pathogenesis.[13] Here we show abundant intercellular and cytoplasmic PAR-2 staining in skin keratinocytes by immunohistochemistry using affinity-purified rabbit antibodies raised against a peptide corresponding to the cleavage site of PAR-2. We also show functional expression of PAR-2 by the skin keratinocyte cell line SVK14 and that activation of this receptor *in vitro* stimulates increased secretion of the

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chemokine IL-8. These data raise the possibility that PAR-2 may play a role in inflammatory skin conditions.

## MATERIALS AND METHODS

*Materials*

The PAR-2 activating peptide, consisting of 11 amino acids with the sequence SLIGKVDGTSH-$NH_2$, was synthesized using standard solid phase 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry with an automated peptide synthesizer (model 432A). Acetylated control peptide was produced using the standard acetic anhydride capping procedure. Peptides were purified by reverse-phase high-performance liquid chromatography and their structure was confirmed by mass spectroscopy as described earlier.[9] RPMI with L-glutamine and fetal calf serum (FCS) were obtained from Gibco (Paisley, UK). Hanks' balanced salt solution without phenol red (HBSS) and Fura-2 acetoxymethyl ester were purchased from Molecular Probes (Eugene, OR, USA). Trypsin and polymyxin B were from Sigma Chemical Co. (Poole, UK). Activated CH–Sepharose 4B was from Pharmacia-LKB Biotechnology (Milton Keynes, UK).

*Preparation of PAR-2 antibodies*

Rabbit polyclonal antibodies were raised to synthetic peptides corresponding to the activation site of human PAR-2 (SKGRSLIGKVDGTSHVTGK-$NH_2$ residues 33–51 of human PAR-2). A multiple antigenic peptide consisting of this sequence was used as an immunogen.[14] Anti-PAR-2 antibodies were purified using an affinity column of the linear peptide coupled to Activated CH–Sepharose 4B as previously described.[15] Preimmune sera was purified using a Protein-A–Sepharose column to yield control IgG.

*Cell culture*

SVK14, a simian virus (SV40)-transformed human skin keratinocyte cell line, was obtained from Professor I. M. Leigh (Cutaneous Research Centre, St. Bartholomew's and the Royal London School of Medicine and Dentistry). Fibroblasts were grown by explant cultures of human oral mucosa. ECV304, a spontaneously transformed human umbilical vein endothelial cell line, and HL60, a promyelocytic leukaemia cell line, were purchased from ECACC (Wiltshire, UK). Cells were cultured at 37°, 5% $CO_2$ in complete medium comprising RPMI-1640 with 25 mM HEPES buffer and 2 mM L-glutamine containing 1% penicillin and streptomycin and 10% heat-inactivated FCS (Gibco).

*Flow cytometric analysis*

SVK14 and fibroblasts were removed using non-enzymatic calcium and magnesium-free HBSS (Cell Dissociation Solution, Sigma). SVK14, fibroblasts and HL60 cells were washed in HBSS and resuspended in $1 \times 10^5$ cells/tube for staining. Indirect immunofluorescence was performed using affinity-purified IgG PAR-2 antibodies (1–20 μg/ml) or preimmune rabbit IgG (1–20 μg/ml) incubated for 20 min at room temperature followed by incubation with fluorescein isothiocyanate (FITC)-labelled swine anti-rabbit antibodies (DAKO, High Wycombe, UK) for 20 min at room temperature. The cells were then analysed on a fluorescence-activated cell sorter (FACScan; Becton Dickinson, Cowley, UK) equipped with Consort 32 Lysys version 1.02 software for data analysis.

*Immunohistochemistry*

Normal skin from breast ($n=3$), abdomen ($n=2$) and buccal mucosa ($n=3$) were stored at −70° before use. Serial sections (5 μm) were cut on a cryostat (Bright, Hemel Hempstead, UK), mounted on Aminopropylethoxysilene (Apes)-coated slides and dried overnight at room temperature. Serial sections were fixed in cold acetone for 1 min before blocking with 10% FCS in 0·2 M Tris–HCl buffer for 15 min to reduce non-specific staining. Rabbit affinity-purified anti-PAR-2 IgG or preimmune control IgG (10 μg/ml) diluted in 2% FCS Tris–HCl were applied to adjacent sections and incubated overnight at 4° in a humid chamber. After washing with PBS, sections were incubated with biotinylated anti-rabbit IgG (DAKO) for 1 hr, followed by avidin–biotinylated alkaline phosphatase complex (DAKO) incubated for 1 hr at room temperature. Colour was developed using Vector Red (Vector Laboratories, Peterborough, UK) containing 1 mM levamisole to block endogenous alkaline phosphate activity. The sections were counterstained with Haemotoxylin, mounted and examined with a light microscope (Photomat FX4, Nikon, Kingston, UK).

*Northern blot analysis*

SVK14, ECV304 and fibroblasts were grown to confluence in T75 flasks in complete medium. After serum starvation for 24 h, total RNA was extracted using Ultraspec (Biotecx, Houston, TX) according to the manufacturer's instructions. Total RNA (20 μg) was electrophoresed in a 1·2% agarose/formaldehyde gel and transferred to Hybond-N nylon membranes (Amersham, Bucks, UK). After fixation by ultraviolet cross-linking (Stratagene, Cambridge, UK), the membrane was hybridized with [$\alpha^{32}$P] dCTP-labelled PAR-2 and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes in QuickHyb solution (Stratagene) containing 100 μl of 10 mg/ml sonicated salmon sperm DNA (Stratagene) for 3 h at 68° and washed in 2 × SSC; 0·1% SDS for 2 × 10 min at room temperature followed by 1 × SSC; 0·1% SDS for 2 × 10 min at 65° and subjected to autoradiography.

*Measurement of intracellular calcium concentration*

SVK14 cells were removed using non-enzymatic dissociation solution and washed twice in an extracellular medium (EM) (25 mM HEPES buffer, pH 7·3, containing 121 mM NaCl, 5·4 mM KCl, 0·8 mM $MgCl_2$, 1·8 mM $CaCl_2$, 6 mM $NaHCO_3$ and 5·5 mM glucose). Cells were counted and resuspended at $6 \times 10^6$ cells/ml, and loaded with 1 mM Fura-2 AM for 30 min at 37° as described previously.[16,17] After centrifugation, cells were resuspended in EM and incubated for 30 min to allow hydrolysis of the intracellular Fura-2 AM. Cells were then centrifuged and resuspended in EM at $2 \times 10^6$ cells/ml for fluorescence measurements in stirred quartz cuvettes. The intracellular calcium concentration ($[Ca^{2+}]_i$) was determined using a Perkin Elmer LS-50 spectrofluorometer. Fura-2 fluorescence was measured at excitation and emission wavelengths of 340 and 510 nm respectively. Loaded cells were maintained at 37° in stirred quartz cuvettes throughout the experiment. After a stable baseline was established, different concentrations of trypsin, PAR-2 agonist peptide, or acetylated control peptide were carefully added to the cells. $[Ca^{2+}]_i$ was calculated using Perkin Elmer LS-50 software together with a dissociation constant for $Ca^{2+}$–Fura-2 of 224 nM.[18]

The increase in intracellular calcium ($\Delta[Ca^{2+}]_i$) showed a hyperbolic dependence on the concentration of trypsin. Data were fitted by non-linear regression to the following equation to obtain estimates for the theoretical maximum increase in intracellular calcium ($\Delta[Ca^{2+}]_{max}$) and the 50% effective concentration ($EC_{50}$) for trypsin:

$$\Delta[Ca^{2+}]_i = \Delta[Ca^{2+}]_{max}[\text{trypsin}]/(EC_{50} + [\text{trypsin}]).$$

*IL-8 immunoassay*

SVK14 cells were seeded at $5 \times 10^4$ cells/well in 24-well plates and cultured at 37°, 5% $CO_2$ in complete medium until confluent and then starved of serum for 24 h before stimulation with various concentrations of trypsin, PAR-2 agonist peptide (SLIGKV-$NH_2$) or acetylated control peptide (Ac-SLIGKV-$NH_2$) in the presence of 10 μg/ml polymyxin B which binds and neutralizes endotoxin. Since the amino terminal serine in SLIGKV-$NH_2$ has been shown to be essential for the agonist activity of the peptide,[3] this serine was acetylated and the peptide Ac-SLIGKV-$NH_2$ was used as a control for any non-specific effects of the peptide. Preliminary kinetic studies at 24-, 48- and 72-h stimulations showed that the maximum concentrations of IL-8 were obtained in the culture medium after 48 h stimulation and thus this time-point was used for more detailed analysis. SVK14 cell culture medium was therefore harvested after 48 h treatment, centrifuged and the supernatants were measured for IL-8 production by enzyme-linked immunosorbent assay (ELISA) (R&D System, Abingdon, UK). After harvesting the culture medium, cells were removed using trypsin (0·1%) and were counted using an automatic cell counter (Coulter, Luton, Beds, UK). Data from three separate experiments were analysed using one-way analysis of variance (ANOVA).

## RESULTS

### PAR-2 flow cytometric analysis of keratinocytes and immunolocalization in skin and mucosa

Polyclonal IgG to PAR-2 were produced in rabbits by using a multiple antigenic peptide[14] corresponding to the activation site of PAR-2 (SKGRSLIGKVDGTSHVTGK-$NH_2$, residues 33–51 of human PAR-2). The resulting antibodies were purified from rabbit serum using an affinity chromatography column consisting of linear peptide SKGRSLIGKVDGTSHVTGK-$NH_2$ coupled to Sepharose as reported previously.[9] In flow cytometric analysis of HL60 and SVK14, SVK14 stained specifically with PAR-2 antibodies and showed clear PAR-2 staining at all of the concentrations used (1, 5, 10 and 20 μg/ml) (Fig. 1). The level of fluorescence intensity obtained with PAR-2 IgG was compared with preimmune rabbit IgG used at the same concentration. By contrast, cells which do not express PAR-2 mRNA such as HL60[2] and fibroblasts[7] did not show specific PAR-2 staining (Fig. 1) (fibroblast data not shown).

The distribution of PAR-2 in skin and buccal mucosa was investigated using immunohistochemistry. As shown in Fig. 2(a), basal and suprabasal keratinocytes in epidermis showed strong staining with PAR-2 antibodies. A similar PAR-2 staining pattern was seen in the non-keratinized epithelium of buccal mucosa. Basal cells showed the highest

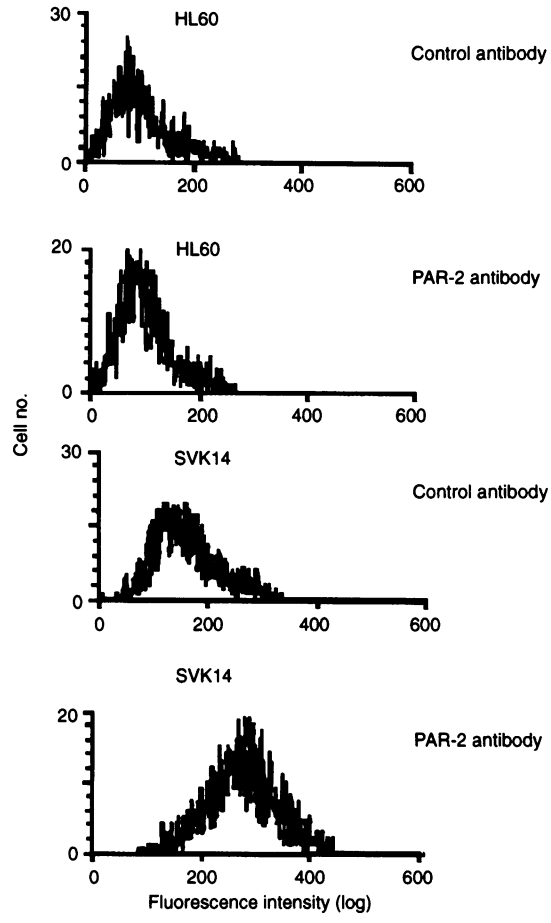


**Figure 1.** Flow cytometric analysis of PAR-2 expression by SVK14 and HL60. Fluorescence intensity is shown on the *x*-axis and cell number on the *y*-axis. The two upper histograms represent the results with HL60 cells. No significant PAR-2 expression was seen with PAR-2 antibody (20 μg/ml) compared with control antibody used at the same concentration. The two lower histograms present results for SVK14 and show clearly, increased fluorescence intensity with PAR-2 antibodies (20 μg/ml).

expression and there was prominent cell membrane and cytoplasmic staining throughout the stratified squamous epithelium (Fig. 2b). Endothelial cells, which were identified in serial sections using factor VIII antibodies (data not shown), also stained positively for PAR-2 in the upper dermis and lamina propria (Fig. 2a,b). Control rabbit IgG, used at the same concentrations, was consistently negative (Fig. 2c).

### Functional expression of PAR-2 by keratinocytes

Total RNA was obtained from SVK14, ECV304 and primary human fibroblasts for Northern blot analysis. The predicted 3·0-kilobase (kb) single transcript for PAR-2 was readily detected in keratinocytes and endothelial cells, with the keratinocyte cell line expressing the highest level of mRNA (Fig. 3). Fibroblasts showed no detectable PAR-2 mRNA. The levels of GAPDH mRNA indicated that similar amounts of total RNA had been loaded. These results were consistent with that shown by Santulli *et al.* using neonatal skin keratinocytes and fibroblasts.[7]

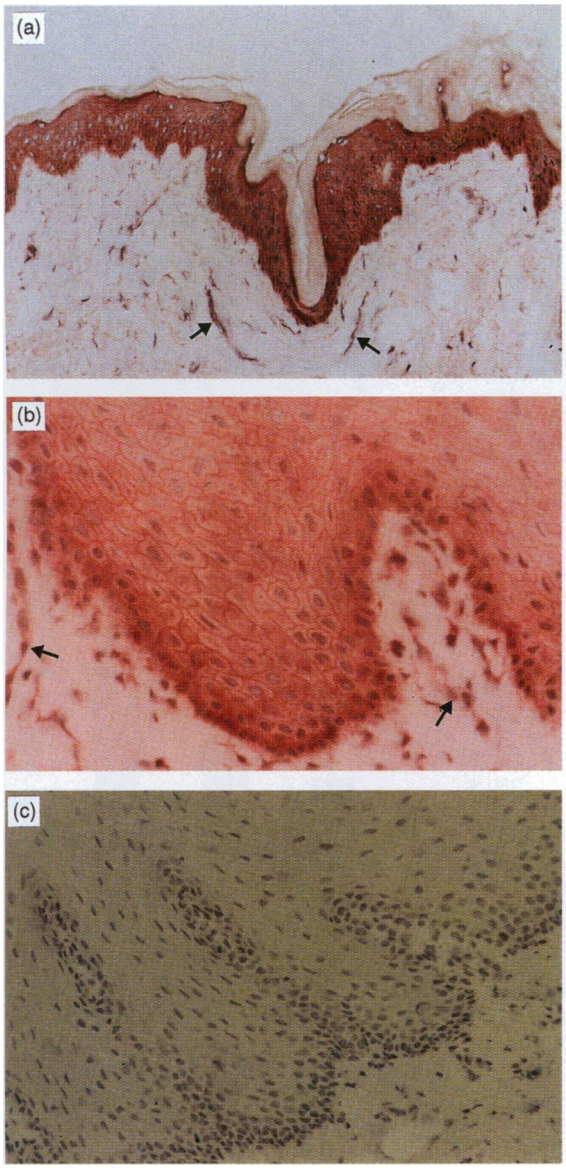


**Figure 2.** Immunohistochemistry with affinity-purified rabbit IgG raised against the extracellular domain of PAR-2 and control IgG. (a) Normal human breast skin showing PAR-2 expression in the epidermis excluding the stratum corneum (×40·6). (b) Human buccal mucosa showing cell membrane and cytoplasmic PAR-2 staining, with the highest staining on basal cells (×81·25). (c) Human buccal mucosa showing consistently negative staining for control IgG (×65·3). PAR-2-positive endothelial cells are indicated by the arrowheads.

We also measured the intracellular calcium flux in SVK14 in response to trypsin or PAR-2 agonist peptide. A representative trace of the magnitude of the $[Ca^{2+}]_i$ in isolated SVK14 in response to trypsin (100 nM) is shown in Fig. 4(a). Trypsin induced a time- and dose-dependent increase in $[Ca^{2+}]_i$, data similar to those shown previously by Santulli *et al.* using primary cultures of neonatal keratinocytes.[7] Figure 4(b) shows the concentration-dependent $[Ca^{2+}]_i$ by SVK14 in response to trypsin (0·05–50 nM). The increase in $[Ca^{2+}]_i$ was dependent on the concentration of trypsin which was analysed according to the equation given above in Materials and Methods. This yielded estimates for the maximum increase in intracellular

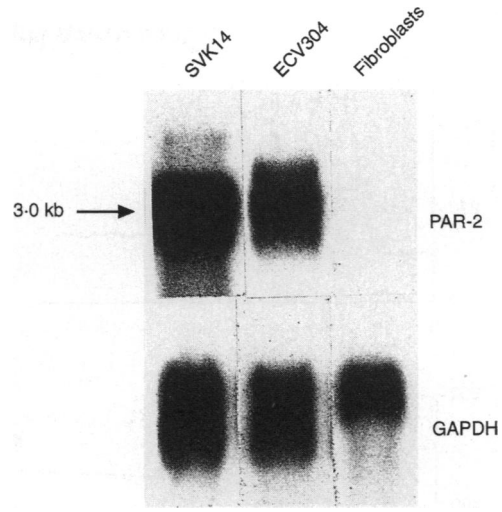


**Figure 3.** Northern blot analysis; total RNA was extracted and probed for human PAR-2 as described in the Materials and Methods. As shown in the upper panel, the predicted 3·0-kilobase transcript of human PAR-2 mRNA is expressed by keratinocytes (SVK14) and endothelial cells (ECV304) but is absent in fibroblasts. The lower panel shows the levels of mRNA encoding GAPDH and confirms that similar levels of total RNA had been loaded.

calcium ($\Delta[Ca^{2+}]_{max}$) of 485 ± 26·6 (nM ± SEM) and an $EC_{50}$ of 1·53 ± 0·32 (nM ± SEM). PAR-2 agonist peptide (200 μM) also induced a time-dependent increase in $[Ca^{2+}]_i$ in SVK14 cells (Fig. 4c), whereas acetylated control peptide did not show a significant increase in $[Ca^{2+}]_i$ by SVK14 (data not shown). However, the magnitude of the $[Ca^{2+}]_i$ in SVK14 cells in response to PAR-2 agonist peptide (200 μM) was much lower than that in response to trypsin (100 nM) (Fig. 4a and 4c).

### PAR-2 activation stimulates IL-8 secretion by keratinocytes

SVK14 cells were stimulated with trypsin or PAR-2 agonist peptide (SLIGKV-$NH_2$) for up to 72 h. Both trypsin and agonist peptide induced a clear concentration-dependent increase in IL-8 secretion by SVK14. Preliminary studies showed that the maximum concentrations of IL-8 were obtained in the culture medium after 48 h of stimulation and this time-point was used for detailed analysis. Trypsin ($10^{-10}$–$10^{-8}$ M) and PAR-2 agonist peptide ($2 \times 10^{-4}$–$8 \times 10^{-4}$ M) caused an increased secretion of IL-8 by SVK14 in a dose-dependent manner. Acetylated control peptide (Ac-SLIGKV-$NH_2$) used at the same concentrations as PAR-2 agonist peptide was without activity. As shown in Fig. 5(a), the highest concentrations of IL-8 were observed in the culture supernatants from cells treated with trypsin at $10^{-9}$ M, approximately 300-fold greater than those obtained from control media (3250 and 11 pg/ml respectively). The PAR-2 agonist peptide ($2 \times 10^{-4}$–$8 \times 10^{-4}$ M) also stimulated IL-8 production, but the levels of IL-8 induced by the agonist peptide were much lower than those induced by trypsin. The highest concentration of peptide used ($8 \times 10^{-4}$ M) induced a response that was about 100-fold greater than resting levels of IL-8 (1013 and 11 pg/ml respectively), and approximately 25-fold greater than those stimulated by acetylated control peptide (1013

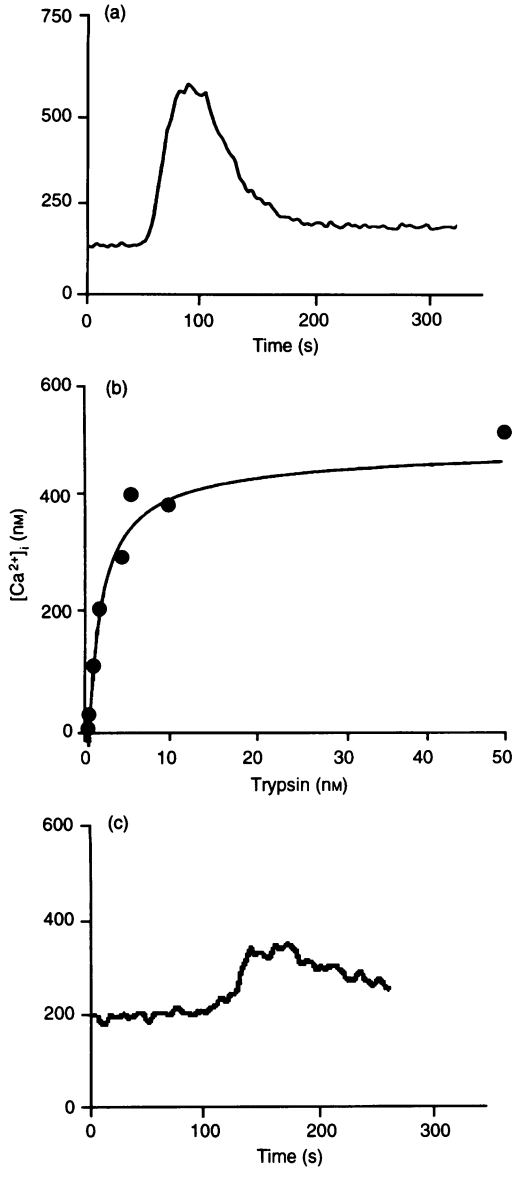


**Figure 4.** $Ca^{2+}$ signalling in SVK14 in response to trypsin or PAR-2 agonist peptide. (a) A single cell suspension was prepared and loaded with Fura-2 and stimulated with 100 nM trypsin. A representative trace of two similar experiments is shown. (b) The increased $[Ca^{2+}]_i$ depends on the concentration of trypsin. SVK14 were loaded with Fura-2 and stimulated with trypsin (0·05–50 nM). The increases in the intracellular calcium ($\Delta[Ca^{2+}]_i$) plotted at different trypsin concentrations represent the mean derived from duplicate experiments. (c) PAR-2 agonist peptide (200 μM) induces a time-dependent increase in $[Ca^{2+}]_i$ in SVK14. A representative trace of two similar experiments is shown.

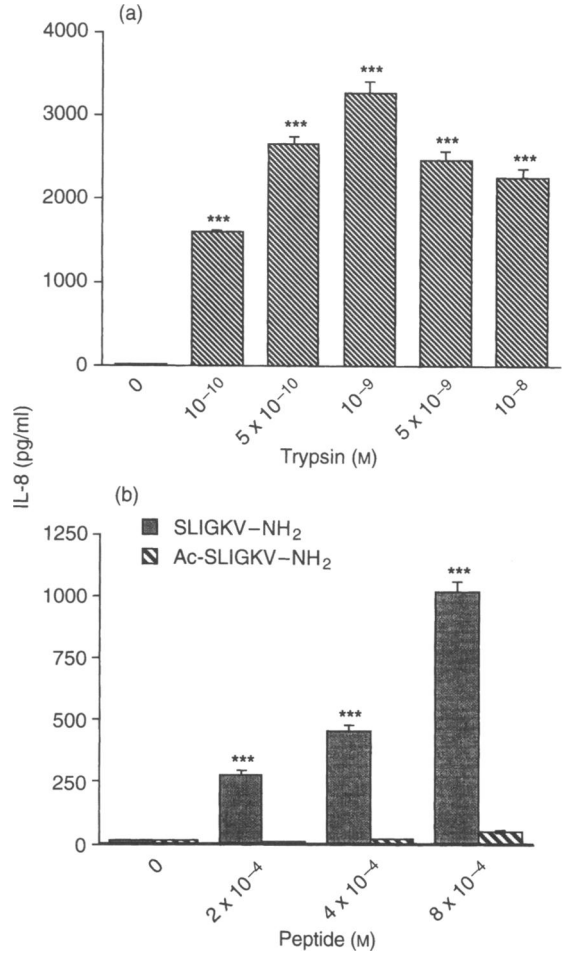


**Figure 5.** Measurement of IL-8 concentrations by ELISA in culture supernatants from SVK14 after PAR-2 activation. IL-8 secretion by SVK14 after 48-h treatment with (a) trypsin ($10^{-10}$–$10^{-8}$ M), or (b) PAR-2 agonist peptide SLIGKV-$NH_2$ or acetylated control peptide Ac-SLIGKV-$NH_2$ ($2 \times 10^{-4}$–$8 \times 10^{-4}$M). PAR-2 activation by trypsin and PAR-2 agonist peptide induced significantly increased levels of IL-8 production. The results shown represent the mean ± SEM obtained from three experiments; ***$P < 0{\cdot}001$.

versus 42 pg/ml) (Fig. 5b). The maximum PAR-2 agonist peptide response at $8 \times 10^{-4}$ M was only comparable to about a third of the maximal trypsin induction at $10^{-9}$ M in the study (1013 versus 3250 pg/ml). SVK14 cell detachment was observed at trypsin concentrations greater than $10^{-9}$ M which may account for the bell-shape curve seen in Fig. 5(a). Cell viability studies confirmed this observation, suggesting that cell death was occurring at higher concentrations of the trypsin used. However, the cell counts obtained with each treatment did not differ significantly.

## DISCUSSION

In this study, we have investigated the expression and function of PAR-2 in keratinocytes. This is the first evidence to show that keratinocytes throughout the whole epidermis, with the exception of the cornified layer, appear to express abundant cell membrane and cytoplasmic PAR-2. A similar staining pattern was found in non-keratinized stratified squamous epithelium of human buccal mucosa, suggesting that PAR-2 expression is not restricted to cornified epithelium. Whether the higher levels of PAR-2 staining seen on basal cells is related to the earlier state of differentiation of these cells remains to be investigated, as does the significance of the cytoplasmic staining, which may reflect receptor internalized after cleavage, or *de novo* production. The PAR-2 staining of endothelial cells is also the first immunohistochemical study

to support the Northern blot analysis shown in this manuscript and a number of reports indicating PAR-2 mRNA expression by these endothelial cells.[6,19,20] The Northern analysis we show confirms the earlier data published by Santulli *et al.* using dermal fibroblasts and neonatal skin keratinocytes,[7] which showed that keratinocytes express PAR-2 mRNA, but fibroblasts do not. However, we have also investigated cell surface expression of PAR-2 by flow cytometric analysis and show clearly that keratinocytes express PAR-2 protein, while HL60 and fibroblasts do not.

Our results demonstrate that PAR-2 activation stimulates IL-8 secretion by keratinocytes. The fact that the agonist peptide (SLIGKV-$NH_2$), which mimics the tethered ligand of PAR-2, stimulated the induction of IL-8 secretion by keratinocytes strongly implicates PAR-2 in this process because the specificity of the agonist peptide was clearly demonstrated by the lack of activity shown by the acetylated peptide, particularly since PAR-2 agonist peptide cannot activate PAR-1 or PAR-3.[3,4] While this paper was in preparation Wakita *et al.* showed that an agonist peptide for murine PAR-2 (SLIGRL-$NH_2$) induced increased gene expression for IL-6 and granulocyte–macrophage colony-stimulating factor (GM-CSF) in cultured human keratinocytes.[21] Therefore, PAR-2 activation appears to signal the up-regulation of a panel of inflammatory cytokines in keratinocytes. However, the work reported by Wakita *et al.*[21] showed that the keratinocytes used in their studies constitutively expressed IL-8 mRNA and therefore they did not examine IL-8 induction in keratinocytes in response to PAR-2 activation. However, our results are supported by Li *et al.* showing that the production of IL-8 by primary cultures of resting skin keratinocytes is very low and often undetectable at the protein level, and can be induced by some inflammatory cytokines.[22]

Since IL-8 is an important chemoattractant for neutrophils and neutrophil accumulation is a prominent feature of psoriasis, the excessive production of IL-8 has been implicated in the pathogenesis.[13] For example an increased level of IL-8 mRNA is found in keratinocytes in the upper epidermis of psoriatic skin.[23] Interestingly, our preliminary immunohistochemical data show that in contrast to normal skin, the intensity of PAR-2 staining appears to be reduced and patchy in suprabasal layers of psoriatic lesional epidermis (data not shown). Whether the reduced expression of PAR-2 is related to receptor synthesis or receptor cleavage is currently under investigation and any potential role of PAR-2 in psoriasis remains to be established.

The results obtained in this study firmly establish that PAR-2 expressed by keratinocytes is functional, since both the agonist peptide for PAR-2 and trypsin induced $Ca^{2+}$ signalling in SVK14 keratinocytes. However, the $Ca^{2+}$ flux induced by agonist peptide appears to occur later than that in response to trypsin, and also the magnitude of $[Ca^{2+}]_i$ response induced by peptide is lower than that by trypsin (Fig. 4a,c) which is consistent with the results shown by Santulli *et al.*[7] Moreover, Wakita *et al.* also showed that SLIGRL increased $[Ca^{2+}]_i$ by skin keratinocytes in cells grown on coverslips, but the effects of SLIGRL were also not as marked as trypsin and the number of cells responding to SLIGRL was lower than that to trypsin.[21]

The reduced potency of agonist peptide in comparison with corresponding enzyme has been found in a number of earlier studies, including thrombin stimulation of fibroblasts[24] and trypsin-induced neutrophil activation.[9] This difference has been attributed to the possibility that the peptide is degraded by aminopeptidases *in vitro*[9] or to the fact that the agonist peptide fails to provide a full signal for reasons of lower avidity or conformational differences.[4] However, an additional explanation is that trypsin may activate receptors other than the cloned protease-activated receptors expressed by keratinocytes. This possibility cannot be excluded, particularly given the fact that unknown protease-activated receptors may exist and also that trypsin may cleave a variety of cell surface proteins containing the trypsin cleavage sequence. In this regard, we have partial sequence data for a candidate PAR-4, which is strongly expressed by keratinocytes (manuscript in preparation). Expression system experiments are in progress to establish whether this new protease-activated receptor is also activated by trypsin.

Surprisingly, Wakita *et al.*[21] showed that PAR-1 is also functional in keratinocyte activation, since thrombin and PAR-1-activating peptide also induced increased $[Ca^{2+}]_i$ and up-regulated IL-6 and GM-CSF gene expression by skin keratinocytes, suggesting that PAR-1 was involved. They showed that keratinocytes expressed both PAR-2 and PAR-1 mRNA by reverse transcription–polymerase chain reaction. In our earlier studies, we also showed that SVK14 keratinocytes expressed PAR-1 mRNA by Northern blot analysis, but the levels of PAR-1 mRNA were much lower than those of PAR-2 and cell surface expression of PAR-1 using flow cytometric analysis of SVK14 keratinocytes was undetectable (manuscript submitted). Moreover, Santulli *et al.*[7] also showed a weaker response of keratinocytes to thrombin in inositol phosphate formation and $Ca^{2+}$ mobilization than that to trypsin, which is consistent with lower levels of PAR-1 expression by keratinocytes shown in our earlier studies. We therefore did not investigate the role of PAR-1 in keratinocytes. Nevertheless, based on the recent report by Wakita *et al.*[21] it is necessary to define clearly whether PAR-1 is involved in keratinocyte activation.

A key question which remains to be answered is the identity of a potential protease ligand for PAR-2 in epidermis. Recently it was shown that mast cell tryptase cleaves and activates PAR-2 in transfected COS-1 cells,[25] but the effects of tryptase on keratinocytes in earlier studies do not provide clear evidence that this is an important ligand for keratinocytes. For example, tryptase stimulates proliferation of primary keratinocytes[26] but a receptor-activating peptide specific for PAR-2 inhibits keratinocyte proliferation.[27] However, a number of serine proteases and protease inhibitors are present in the epidermis of skin in health and disease[10,11] and it is possible that the protease ligands for PAR-2 may be among these, produced within the epidermis, perhaps by keratinocytes. Certainly the full physiological and pathological significance of PAR-2 in keratinocyte biology awaits the identification of the relevant protease ligand.

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