ORIGINAL ARTICLE Basic Mechanisms in Allergic Disease

The role of protease-activated receptors PAR-1 and PAR-2 in the repair of 16HBE 14o[−] epithelial cell monolayers *in vitro*

D. Ewen¹, S.L. Clarke¹, J.R. Smith¹, C. Berger², G. Salmon², M. Trevethick² and J.K. Shute¹¹Institute of Biomedical and Biomolecular Sciences, University of Portsmouth, Portsmouth, Hampshire, UK and ²Allergy and Respiratory Biology, Pfizer Global Research and Development, Sandwich, UK

Clinical & Experimental Allergy

Summary

Background We recently reported that repair following mechanical wounding of epithelial cell layers *in vitro* is dependent on fibrin formation and the activity of locally expressed coagulation cascade proteins. Serine proteases of the coagulation cascade are an important group of protease-activated receptor (PAR) activators and PAR-1 to 4 are expressed by the normal bronchial epithelium.

Objective We tested the hypothesis that activation of PAR-1 and PAR-2 by coagulation cascade proteases stimulates epithelial repair via effects on fibrin formation.

Methods Using mechanically wounded 16HBE 14o[−] epithelial cell layers in culture, we investigated the effect of PAR-1 and PAR-2 agonist peptides, control partially scrambled peptides and PAR-neutralizing antibodies on the rate of repair and fibrin formation. Coagulation factors in culture supernatants were measured by immunoblot. RT-PCR was used to investigate PAR-1, PAR-2 and PGE2 receptor (EP-1 to EP-4) expression in this model and qRT-PCR to quantify responses to wounding. Additionally, we investigated the effect of exogenously added factor Xa (FXa) and neutrophil elastase and the influence of PGE2 and indomethacin on the repair response.

Results PAR-1 and PAR-2 peptide agonists stimulated the rate of repair and enhanced the formation of a fibrin provisional matrix to support the repair process. Conversely, PAR-neutralizing antibodies inhibited repair. Under serum-free culture conditions, 16HBE 14o[−] cells expressed EP-2 and EP-3, but not EP-1 or EP-4, receptors. Wounding induced an increased expression of EP-3 but did not alter EP-2, PAR-1 or PAR-2 expression. In the absence of PAR agonists, there was no evidence for a role for PGE2 in fibrin formation or the repair process. Indomethacin attenuated fibrin formation in wounded cultures only in the presence of the PAR-2 peptide. FXa stimulated epithelial repair while neutrophil elastase reduced the levels of coagulation factors and inhibited repair.

Conclusion Locally expressed serine proteases of the coagulation cascade activate PAR-1 and PAR-2 to enhance fibrin formation and bronchial epithelial repair.

Keywords bronchial epithelium, coagulation, elastase, factor Xa, fibrin, protease-activated receptor

Submitted 12 August 2008; revised 9 December 2009; accepted 10 December 2009

Correspondence:

Dr Janis K. Shute, School of Pharmacy and Biomedical Sciences, St Michael's Building, White Swan Road, Portsmouth PO1 2DT, Hampshire, UK.

E-mail: jan.shute@port.ac.uk

Cite this as: D. Ewen, S.L. Clarke, J.R. Smith, C. Berger, G. Salmon, M. Trevethick and J.K. Shute, *Clinical & Experimental Allergy*, 2010 (40) 435–449.

Introduction

The normal airway epithelium provides a physical barrier to the invasion of lung tissue by inhaled foreign particles, including pollutants, allergens, bacteria and viruses. Together with chemical and cellular mechanisms of the innate immune system, this physical barrier maintains sterility in the lower airways [1, 2]. Proteolytic activity

associated with inhaled mites, fungi, pollen and bacteria damages the airway epithelium [3–5] and induces an inflammatory response that contributes to the development of a number of respiratory diseases, including asthma and rhinitis, in susceptible individuals, but not in healthy subjects with the same level of exposure [5]. Despite the potential of inhaled particles to damage the airway epithelium, the normal bronchial epithelium

remains relatively intact and continuous repair of this tissue is indicated. Animal models have demonstrated that bronchial epithelial repair is dependent on the rapid formation of a fibrin gel [3] and it was postulated that this was entirely dependent on plasma-derived coagulation factors [6]. However, we recently reported that repair following mechanical wounding of bronchial epithelial cell layers *in vitro* is similarly dependent on rapid fibrin formation, but critically dependent on the activity of locally expressed coagulation cascade proteins [7].

The protease-activated receptors (PARs) are a family of four widely expressed G-protein coupled receptors (PAR-1 to -4) activated by the proteolytic removal of an inhibitory amino terminus by cleavage at a site specific to each receptor [8, 9]. Cleavage of the N-terminus exposes a new N-terminal tethered ligand able to bind to another site on the molecule and activate the receptor. Serine proteases of the coagulation cascade are an important group of PAR activators and, for example, thrombin is an activator of PAR-1, PAR-3 and PAR-4, factor Xa (FXa) activates PAR-1 and PAR-2 and FVIIa activates PAR-2 [10]. Other important activators of PAR-2 include mast cell tryptase, trypsin and the serine proteases associated with house dust mite allergens Der p3 and Der p9 [5, 11, 12]. Conversely, neutrophil-derived proteases, neutrophil elastase, cathepsin G and proteinase 3 can inactivate PAR-1 and PAR-2, disarming them by the cleavage of the N-terminal exodomain downstream from the activation site [13, 14].

PARs 1–4 are expressed by the normal bronchial epithelium, with an apical distribution of PAR-1 and PAR-3 and a more diffuse distribution of PAR-2 and PAR-4 [15, 16]. PARs on the airway epithelium translate endogenous and exogenous proteolytic activity in epithelial lining fluid into both pro-inflammatory [5] and anti-inflammatory bronchoprotective signals [16, 17]. PAR-1- and PAR-2-activating peptides have been shown previously to stimulate primary and A549 bronchial epithelial cell lines to synthesize PGE2 [18]. Additionally, PGE2 stimulates the repair response of mechanically wounded 16HBE 140[−] bronchial epithelial cells in culture [19]. In particular, the PAR-2-PGE2-prostanoid EP receptor axis has been proposed to have anti-inflammatory and bronchoprotective effects in the airway [20].

We, therefore, tested the hypothesis that activation of PAR-1 and PAR-2 expressed by the 16HBE 140[−] bronchial epithelial cell line stimulates epithelial repair via effects on fibrin formation.

In summary, we investigated the effect of a PAR-1 agonist peptide, TFRIFD-NH₂ and control partially scrambled peptide FTRIFD-NH₂, and a PAR-2 agonist peptide, SLIGKVD-NH₂ and control partially scrambled LSIGKVD-NH₂, on fibrin formation and the repair of wounded 16HBE 140[−] epithelial cell layers in culture. We investigated the expression of PAR-1 and PAR-2 in this model, the effect of PAR-neutralizing antibodies and the effect of the exogenously added proteases, FXa and neu-

trophil elastase. Additionally, we investigated the expression of PGE2 receptors (EP-1 to EP-4) and the influence of PGE2 and indomethacin on the repair response.

Methods

Culture of 16HBE 140[−] cells

16HBE 140[−] cells were cultured for 48 h in 24-well plates as described previously [7] until fully confluent. At confluence, cells were washed with PBS and quiesced overnight in serum-free MEM-ITS [basal MEM containing 2 mM L-glutamine and antibiotic/antimycotic (100 U/mL penicillin G, 100 µg/mL streptomycin, 250 ng/mL amphotericin B (antibiotic–antimycotic liquid supplement (× 100) (Invitrogen Ltd, Paisley, Renfrewshire, UK)] supplemented with 10 µg/mL bovine insulin, 5.5 µg/mL human transferrin and 5 ng/mL sodium selenite [ITS liquid media supplement (× 100) (Sigma, Dorset, UK)]. Before each experiment, cells were washed by adding 500 µL per well of phosphate-buffered saline (PBS, Invitrogen Ltd), and 250 µL of fresh, serum-free MEM-ITS was added to each well.

Stimulation of 16HBE 140[−] cells with protease-activated receptor peptide agonists

We used synthetic activating peptides at a concentration shown previously to induce the release of cytokines and PGE2 in primary and human bronchial epithelial cell lines [18]. Synthetic PAR-1 peptide agonist (TFRIFD-amide), PAR-2 peptide agonist (SLIGKVD-amide) and the corresponding PAR-1 control peptide with the first two amino acids in reverse order (FTRIFD-amide) and PAR-2 control peptide (LSIGKVD-amide), were prepared as a 10 mM stock solution in 1% DMSO and added to cells in a final concentration of 400 µM in a volume of 250 µL per well and the plate was incubated for 30 min at 37 °C. Peptides were synthesized by Peptide Protein Research Ltd (Hampshire, UK). A final concentration of 0.04% DMSO was also included as a control. Wound repair was carried out as described below.

The effect of protease-activated receptor neutralizing antibodies

16HBE 140[−] cell layers were treated with PAR-1- (mouse monoclonal ATAP2) and PAR-2 (mouse monoclonal SAM11)-neutralizing antibodies (Santa Cruz Biotechnology, supplied by Insight Biotechnology Ltd, Wembley, UK) or normal mouse IgG (Sigma) at 20 µg/mL for 60 min before wounding.

Kinetic analysis of wound repair of 16HBE 140[−] cells in the presence of protease-activated receptor-activating peptides

Cells were mechanically wounded with a P2 Gilson pipette tip, one horizontal scrape per well. Immediately after

wounding, the plate was placed on the heated stage at 37 °C of the inverted wide field kinetic video microscope (Zeiss Axiovert 200m; Carl Zeiss Ltd, Hertfordshire, UK) and Velocity[®] software was used to take images of the same section of wound for 13 h, with time intervals of 15 min. Velocity[®] software was also used to analyse wound repair by taking 10 measurements across the wound at each time-point up to 13 h.

Effect of protease-activated receptor-activating and control peptides on fibrinogen, factor XIIIa and D-dimer expression following wounding

Synthetic PAR-1 and PAR-2 peptide agonists and control peptides were added to cells at a final concentration of 400 µM, in a volume of 250 µL per well and the plate was incubated for 30 min at 37 °C. For cells that were exposed to a PAR peptide agonist in the presence of indomethacin, cells were pre-treated with indomethacin at a final concentration of 10 µM for 30 min at 37 °C before the addition of the PAR peptide agonist. Cells were mechanically wounded with a P2 Gilson pipette tip in a cross-hatch pattern of 2, 4 or 8 scrapes (W2, W4 and W8) and incubated at 37 °C for 2 h. Supernatants were harvested, cleared by centrifugation and stored in aliquots at –80 °C before analysis of fibrinogen, FXIIIa and for D-dimers by immunoblot, as described previously [7]. In view of the wide tissue distribution of tissue transglutaminase, it has been confirmed that the FXIIIa antibodies used in this study do not cross-react with purified tissue transglutaminase as determined by a direct ELISA (Hugh Hoogendoorn, President, Affinity Biologicals, personal communication).

Immunohistochemical analysis of protease-activated receptor-1 and protease-activated receptor-2 expression

16HBE 140[–] cells were grown to confluence and where indicated NE was added at 0.3 U/mL (200 nM) for 30 min. Cells were wounded and either fixed immediately or incubated for further 2 h. Cells were fixed in 4% w/v paraformaldehyde in PBS for 30 min, washed in PBS and permeabilized with ice-cold methanol for 5 min at –20 °C. Cells were washed in PBS and incubated overnight in PBS containing 1% w/v bovine serum albumin at 4 °C to block non-specific binding sites. Cells were incubated with primary PAR-1 and PAR-2 antibody (Santa Cruz Biotechnology, as above) at 1 µg/mL in PBS containing 1% BSA for 1 h at room temperature, washed five times for 5 min each with PBS containing 0.02% Tween-20, and the secondary antibody, goat anti-mouse Alexa Fluor 488 F(ab')₂ fragment (Invitrogen Ltd) added at 1.5 µg/mL for 1 h at room temp and the washing steps repeated. The slides were mounted using a Vectashield mounting medium (Vector Labs, Peterborough, UK) and viewed using a Zeiss LSM 510 confocal microscope.

Receptor mRNA expression

Total RNA isolation for reverse transcriptase-polymerase chain reaction (RT-PCR). 16HBE 140[–] cells were seeded at a density of 400 000 cells per well in six-well plates and cultured for 48 h until fully confluent. At confluence, cells were quiesced overnight. Before the experiment, MEM-ITS was refreshed. Cells were maximally wounded with four horizontal scrapes and four vertical scrapes and subsequently incubated for 2, 4, 6, 8 and 10 h at 37 °C. Cells were washed and harvested by trypsinization and centrifugation. To each tube, 600 µL of pre-chilled denaturing solution (Promega, Southampton, UK) was added and the mixture homogenized (Ultra-Turrax T25, Janke & Kunkel IKA Labortechnik, Staufen, Germany) at 24 000 r.p.m for 1 min at room temperature. Total RNA was extracted from the homogenate with phenol:chloroform:isoamyl alcohol (99:24:1, pH 4.7) (Promega) and precipitated from the upper phase with an equal volume of isopropanol at –20 °C for 30 min. The RNA was recovered by centrifugation at 10 000 g for 20 min at 4 °C, washed with ice-cold 75% v/v ethanol and air dried in an RNase-free environment for 15 min. RNA was dissolved in nuclease-free water and RNA concentration and purity were estimated by optical density determination. The 260/280 ratio was usually > 1.6, indicating suitable purity of RNA.

Primer design. The 'Blast' tool was used to design a forward and reverse primer pair (Table 1) against a region of the gene in question.

One-step reverse transcriptase-polymerase chain reaction for protease-activated receptor-1, PAR-2 and β-actin. A single host mix of 50 µL was created, consisting of 25 µL of two-times concentrated reaction mix (Invitrogen), 1 µL

Table 1. Forward and reverse primer sequences used for RT-PCR

Gene	Direction	Sequence	Product size (bp)	T _m (°C)
β-actin	Forward	gcgggaaatcgtgctgacatt	232	57.0
	Reverse	gatggagtgaaggtagtttcgtg		56.0
PAR-1	Forward	ggatatttgaccagctcctgg	400	54.0
	Reverse	agatggccagacaagtgaagg		54.0
PAR-2	Forward	ctgcattctgctcactgga	400	54.0
	Reverse	attgccaggagatgccaatg		54.0
EP-1	Forward	gcgctgcccattcttccatga	448	59.0
	Reverse	tgccccgggtactcgactcata		59.0
EP-2	Forward	tctcctgttccactgtctg	446	54.0
	Reverse	gagcaccgagacaatgagaa		52.0
EP-3	Forward	atgaaggagaccggggctacgga	515	63.0
	Reverse	cgcgtcttcattgtgctgcatac		61.0
EP-4	Forward	gcgagtacagcaccttcatt	543	52.0
	Reverse	agatgaccattctggatctcg		52.0

PAR, protease-activated receptor; RT-PCR, reverse transcriptase polymerase chain reaction.

of forward (0.1 µg/mL) and 1 µL of reverse (0.1 µg/mL) primers for PAR-1 and PAR-2 (Sigma-Genosys Ltd, Cambridge, UK), 1 µL of SuperScriptTM II RT/platinum[®] Taq Hi Fi Mix, 1 µL of template RNA (1 µg) and 21 µL of nuclease-free water. RT-PCR reactions were subjected to 50 °C for 30 min to synthesize the cDNA, then pre-denaturation for 2 min at 94 °C, followed by 40 cycles of amplification (PAR-1 and PAR-2) or 30 cycles of amplification (β-actin) consisting of denaturation at 94 °C for 15 s, annealing at 60 °C for 20 s and extension for 45 s at 68 °C. This was followed by a final extension step of 10 min at 72 °C.

Under these conditions, the amplification of each product and the internal standard was linear.

One-step reverse transcriptase-polymerase chain reaction for EP-1–4. A single host mix of 50 µL was created, consisting of 25 µL of two-times concentrated reaction mix (Invitrogen), 1 µL of forward (0.1 µg/mL) and 1 µL of reverse (0.1 µg/mL) primers for EP-1 (Sigma-Genosys Ltd), 1 µL of SuperScriptTM II RT/platinum[®] Taq Hi Fi Mix, 1 µL of template RNA and 21 µL of nuclease-free water. RT-PCR reactions were subjected to 50 °C for 30 min to synthesize the cDNA, then pre-denaturation for 2 min at 94 °C, followed by 40 cycles of amplification consisting of denaturation at 94 °C for 15 s, annealing at 64 °C for EP-1, 56 °C for EP-2, 66 °C for EP-3 and 57 °C for EP-4, for 20 s and extension for 45 s at 68 °C. This was followed by a final extension step of 10 min at 72 °C.

Under these conditions, the amplification of each product was linear.

Analysis of reverse-transcriptase polymerase chain reaction products by agarose gel electrophoresis. Products were analysed on a 2% (w/v) agarose (BDH) gel containing 0.0001% v/v ethidium bromide alongside a 100 bp DNA marker ladder (Invitrogen). Bands were visualized by ethidium bromide fluorescence under ultraviolet light. Densitometry was carried out using SynGene software, to semi-quantify the relative abundance of mRNA bands encoding each gene sequence compared with the internal control, β-actin.

Quantitative real-time reverse-transcriptase polymerase chain reaction. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was used to confirm the absence or presence of changes in the expression of PAR-1, PAR-2, EP-2 and EP-3 receptors. Cells were cultured as described above, and the receptor expression was determined in the absence and presence of PAR-1- and PAR-2-activating peptides (400 µM) added for 30 min before wounding the cells mechanically, as described above. RNA was isolated at 6- and 20-h post-wounding using the RN-easy (Qiagen, Crawley, UK) and cDNA made using the Superscript III platinum kit (Invitrogen). The samples were analysed using a 7900HT TaqMan real-time PCR system (Applied Biosystems, Warrington, UK).

Sequences of the primers used for qRT-PCR (Applied Biosystems)

PAR-1 Forward:	cggtttctggctgtggtgta
PAR-2 Forward:	ctccaggaagaaggcaacattg
EP-2 Forward:	gggcacccctacttctacca
EP-3 Forward:	gggctctcctcgttggtcat
PAR-1 Reverse:	aaggaagccctcccagagta
PAR-2 Reverse:	gggatggtagaccagcagaat
EP-2 Reverse:	agcagaagagcaggagact
EP-3 Reverse:	tgcggcgccctgat

Immunoassay for interleukin-8. 16HBE 140[−] cells were seeded at a density of 400 000 cells per well in 6-well plates and cultured for 48 h until fully confluent. At confluence, cells were quiesced overnight in serum-free MEM-ITS. Cell layers were maximally wounded with four horizontal scrapes and four vertical scrapes and incubated for 12 h at 37 °C. The supernatants were harvested, cleared by centrifugation and stored at −80 °C. IL-8 was analysed using a Pelikine CompactTM human IL-8 ELISA kit (Mast Diagnostics, Liverpool, UK) as described by the manufacturers. IL-8 in cell culture supernatants diluted 1 in 10 with MEM-ITS was assayed against a standard IL-8 concentration curve prepared in the range of 1, 2.5, 6.1, 15.4, 38.4, 96 and 240 pg/mL.

Immunoassay for prostaglandin E2 metabolites. The prostaglandin E metabolite kit (Cayman Chemical Co., Ann Arbor, MI, USA) was used according to the manufacturer's instructions to measure metabolites of PGE2 in culture supernatants from wounded cell layers in the absence and presence of PAR-1 and PAR-2 peptides.

Effect of indomethacin on the repair of 16HBE 140[−] monolayers. A concentration range of 0, 0.01, 0.1, 1, 3 and 10 µM indomethacin (1 mM stock dissolved in 1% DMSO: Sigma) was added to cells in a volume of 250 µL per well and the plate was incubated for 30 min at 37 °C. A concentration range of up to 0.01% DMSO, corresponding to 10 µM indomethacin was also included as the vehicle control. Analysis of wound repair was then carried out as described below.

Effect of prostaglandin E2 on repair of 16HBE 140[−] monolayers. A concentration range of 0, 0.1, 1, 5, 10 and 20 µg/mL PGE2 (2 mg/mL stock dissolved in 50% ethanol/50% MEM-ITS: Sigma) was added to cells in a volume of 250 µL per well and the plate was incubated for 30 min at 37 °C. Ethanol controls at a final concentration of 0.0025%, 0.025%, 0.125%, 0.25% and 0.5% corresponding to 0.1, 1, 5, 10 and 20 µg/mL PGE2, respectively, were also included. Analysis of wound repair was then carried out as described below.

Stimulation of 16HBE 14o⁻ cells with exogenous factor Xa. A concentration range of: 0, 50, 100 and 200 nM FXa (20 mM stock dissolved in PBS: Calbiochem[®], supplied by Merck Chemicals Ltd., Nottingham, UK) was added to cells in a volume of 250 µL per well and the plate was incubated for 30 min at 37 °C. Analysis of wound repair was then carried out as described below.

Effect of neutrophil elastase on the repair of 16HBE 14o⁻ monolayers. A concentration range of 0, 0.1, 0.03, 0.1, 0.3 and 0.5 U/mL neutrophil elastase (3 U/mL stock dissolved in 1 × PBS: Sigma) was added to cells in a volume of 250 µL per well and the plate was incubated for 30 min at 37 °C. Analysis of wound repair was then carried out as described below.

Wound repair of 16HBE 14o⁻ cells in the presence of factor Xa, neutrophil elastase, indomethacin and prostaglandin E2. Cells were mechanically wounded with a P2 Gilson pipette tip, one horizontal scrape per well. Immediately after wounding, one photograph per well was taken of a representative section of wound using the inverted microscope (Leica Microsystems, Wetzlar, Germany) and a Ricoh XR-3000 35 mm camera. Cells were returned to the incubator, at 37 °C for 13 h. A second set of photographs was then taken of the same section of wound. Photographs were developed and wound repair was analysed by taking 10 measurements across the wound and the % original wound width was calculated at 13 h. Supernatants and cells were harvested and stored at -80 °C, as described above.

Stimulation of 16HBE 14o⁻ cells with neutrophil elastase. A concentration range of 0, 0.1, 0.03, 0.1, 0.3 and 0.5 U/mL neutrophil elastase (3 U/mL stock dissolved in PBS: Sigma) was added to cells in a volume of 250 µL per well and the plate was incubated for 30 min at 37 °C. Cells were then mechanically wounded (W8) as described above and incubated for 20 min and 2 h at 37 °C. Cell culture supernatants were then harvested at each time-point, as described above, and the concentration of fibrinogen and FXIII was determined by immunoblot as described previously [7].

Lactate dehydrogenase (LDH) assay. The LDH assay (TOX-7, Sigma) was used to determine whether the effects of neutrophil elastase on wound repair and coagulation factors release were due to cytotoxicity. The assay was adapted for use in a 96-well plate. Cell culture supernatants and cell lysates were diluted 1 in 10 by adding 5 µL of sample to 45 µL of PBS. The LDH assay solution was prepared by mixing equal volumes of LDH substrate, LDH enzyme and LDH tetrazolium dye. To each well, 25 µL of the LDH assay mixture was added. The plate was incubated for 5 min at room temperature, protected from light. In order to stop the reaction, 8.33 µL of 1 M HCl was added

to each well and the absorbance at 490 nm was measured using a microplate reader (Dynex MRX microplate reader, West Sussex, UK).

Statistical analysis

Statistical analysis was carried out using the GraphPad Prism 4 software. The effect of wounding on receptor expression and coagulation factor concentration in culture supernatants was analysed by one-way ANOVA and Dunnett's *post hoc* test. Data on the effect of peptides under each condition were analysed by two-way ANOVA and Bonferroni *post hoc* tests.

Results

The effect of protease-activated receptor-1- and protease-activated receptor-2-activating peptides on wound repair

Using video kinetic analysis, initial experiments found no effect of PAR-1- and PAR-2-activating peptides added at a final concentration of 100 µM on wound repair. However, the data in Fig. 1 show that both PAR-1- and PAR-2-activating peptides added at a final concentration of 400 µM significantly increase the rate of repair of bronchial epithelial cell monolayers, compared with the untreated wells and wells treated with control peptides. With respect to the untreated cell cultures, the mean wound width at 13 h

Fig. 1. Effect of a PAR-1 (a) and a PAR-2 (b) peptide agonist on the rate of wound repair of 16HBE 14o⁻ cell layers. CP denotes control peptide. Data represent mean ± SEM of three independent experiments. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 compared with no drug control.

was $263.58 \pm 14.82 \mu\text{m}$ compared with $768.28 \pm 19.89 \mu\text{m}$ at 0 h, indicating 34.3% of the original wound width remained open. The PAR-1 and PAR-2 control peptides had no effect on wound repair compared with the no drug control wells. However, both PAR-1 and PAR-2 peptide agonists significantly enhanced wound repair. Wound repair was complete 12 h following the addition of the PAR-1 peptide agonist, and 11 h following the addition of the PAR-2 peptide agonist. The effect of the PAR-2 agonist was significantly ($P < 0.05$) greater than that of PAR-1 agonist between the time-points of 3-h and 10-h post-wounding.

Conversely, in separate experiments, antibodies against amino acids 42–55 of human PAR-1 (ATAP2) and amino acids 37–50 of human PAR-2 (SAM11) significantly inhibited the repair response. The PAR-1 antibody reduced the initial rate of wound repair from 130.6 ± 5.87 to $92.4 \pm 16.3 \mu\text{m/h}$ ($P = 0.03$, $n = 3$), and wound closure from $98.3 \pm 1.25\%$ to a maximum of $65.1 \pm 4.7\%$ at 10-h post-wounding ($P < 0.0001$, $n = 3$). Similarly, the PAR-2 antibody reduced the initial rate of wound repair from 130.6 ± 5.87 to $96.7 \pm 9.5 \mu\text{m/h}$ ($P = 0.01$, $n = 5$), and wound closure from $98.32 \pm 1.25\%$ to $73.6 \pm 7.1\%$ ($P < 0.0001$, $n = 3$). In the presence of either of the antibodies, the wounds remained open even after prolonged overnight incubation. Normal mouse IgG had no significant effect on any parameter.

Protease-activated receptor-1 and protease-activated receptor-2 expression in response to wounding

PAR-1 and PAR-2 were both constitutively expressed by 16 HBE cells, as reported previously [21]. Semi-quantitative RT-PCR indicated that their expression was not increased with wounding at any time-point (fig. 2), and

this was confirmed by quantitative real-time RT-PCR. qRT-PCR analysis also demonstrated that the presence of PAR-activating peptides in the cultures did not increase PAR1 or PAR-2 expression.

Immunohistochemical analysis demonstrated the constitutive expression of both PAR-1 and PAR-2 receptors at the protein level (supporting information Figs S1 and S2). PARs were distributed throughout the cytoplasm and on the cell membrane. In the wounded cultures, we observed a tendency to increased fluorescence in the cells at the wound edge, although this was not consistently seen between cultures or even along the length of a single wound, and was not quantifiable. There was also no clear

Fig. 2. (a). Representative gel illustrating the effect of time post-wounding on the mRNA expression of PAR-1 in 16HBE 140⁺ cells. 2% (w/v) agarose gel electrophoretic separation of PAR-1 amplicons generated by RT-PCR from the total RNA extracted from 16HBE 140⁺ cells at various time-points following mechanical wounding. Lane contents as follows: BP, 100 bp ladder; BL, baseline (unwounded cells); 2 h, 2-h post-wound; 4 h, 4-h post-wound; 6 h, 6-h post-wound; 8 h, 8-h post-wound; 10 h, 10-h post-wound. (b) Effect of wounding on mRNA levels of PAR-1 in 16HBE 140⁺ cells. Data bars indicate the ratio of PAR-1 to β -actin internal control. BL, baseline i.e. unwounded cells. Data represent mean SEM of three independent experiments. (c) Representative gel illustrating the effect of time post-wounding on the mRNA expression of PAR-2 in 16HBE 140⁺ cells. 2% w/v agarose gel electrophoretic separation of PAR-2 amplicons generated by RT-PCR from the total RNA extracted from 16HBE 140⁺ cells at various time-points following mechanical wounding. Lane contents as follows: BP, 100 bp ladder; C, negative control (no RNA); BL, baseline (unwounded cells); 2 h, 2-h post-wound; 4 h, 4-h post-wound; 6 h, 6-h post-wound; 8 h, 8-h post-wound; 10 h, 10-h post-wound; PI, Platelet RNA (positive control). (d) Effect of wounding on mRNA levels of PAR-2 in 16HBE 140⁺ cells. Data bars indicate the ratio of PAR-2 to β -actin internal control. BL, baseline i.e. unwounded cells. Data represent mean $\pm$ SEM of three independent experiments.

evidence of receptor redistribution to the cell membrane following wounding.

The effect of protease-activated receptors on coagulation factor release

In view of the role of fibrin formation in wound repair [7], we investigated the effects of PAR activation on the release of the coagulation factors fibrinogen and FXIII into cell culture supernatants. If the increased rate of wound repair in response to PAR-1 and PAR-2 agonists was occurring via enhanced fibrin formation, it was expected that the concentration of D-dimer fragments in cell culture supernatants would be increased. PGE2 has also been shown to be a product of PAR-activated bronchial epithelial cells [18] and to stimulate bronchial epithelial wound repair [19]. Cells were therefore pre-treated with the non-specific COX inhibitor indomethacin, in order to determine whether the effect of PAR-1 and PAR-2 agonists on the coagulation factor release was mediated *via* prostanoid production.

Figure 3 illustrates the effect of wounding, and the effect of PAR-1 and PAR-2 peptides on confluent and wounded cultures, in the absence and presence of indomethacin, on the release of fibrinogen (Fig. 3a), FXIII (Fig. 3b) and the formation of D-dimers as a marker of fibrinogenesis and fibrinolysis (Fig. 3c).

As reported previously [7], mechanical wounding of confluent 16HBE monolayers significantly (see Table 2) increased the concentration of fibrinogen, FXIII and D-dimers in the culture supernatant. Pre-treatment of the monolayers with indomethacin before wounding had no significant effect on levels of any coagulation factor, indicating that prostanoids were not involved in the response. However, the PAR-1 and PAR-2 peptide agonists had strong stimulatory effects on the release of fibrinogen and FXIII, and D-dimer formation, in both confluent and wounded cell cultures. In the confluent monolayers, the magnitude of the response to the PAR-2 peptide agonist was greater than that for the PAR-1 agonist, significantly so for both fibrinogen and D-dimer levels. The control peptides, which had the first two amino acids in reverse order, did not significantly enhance coagulation factor levels in the supernatants, indicating that the agonist peptides were activating the appropriate receptors. Conversely, the presence of the control peptides partially, but significantly *inhibited* the increase in levels of coagulation factors in culture supernatants in response to wounding (Table 2). Indomethacin had no effect on the coagulation factor levels stimulated by the PAR-1 agonist in either confluent or wounded cell layers. However, a significant inhibitory effect of indomethacin on the PAR-2 stimulated increase in FXIII was observed (Fig. 3b) in wounded cell cultures only, which translated into reduced D-dimer levels in the same culture supernatants (Fig. 3c).

The effect of prostaglandin E2 and indomethacin on wound repair and the release of coagulation factors

In view of reports that PGE2 enhances bronchial epithelial repair [19] and PAR agonists stimulate human bronchial epithelial cells to produce PGE2 [18], we investigated the effect of exogenously added PGE2 on wound repair. Under the conditions used, PGE2 in the concentration range 0.1–20 µg/mL did not stimulate repair of wounded 16HBE cell monolayers over 13 h ($n = 3$, data not shown). In addition, indomethacin in the concentration range 0.01–10 µM had no effect over 13 h, indicating no role for endogenous PGE2, or other prostanoids, in the repair response ($n = 3$, data not shown).

Similarly, PGE2 in the concentration range of 1–20 µg/mL did not significantly affect the fibrinogen, FXIII or D-dimer concentration in confluent or wounded cell cultures ($n = 3$, data not shown).

Expression of prostaglandin receptors in response to wounding

Considering previous reports, the lack of effect of exogenous PGE2 was somewhat surprising; hence, we examined prostaglandin receptor expression under our serum-free culture conditions. We detected no expression of EP-1 or EP-4 receptors, which are the receptors reported to be involved in bronchial epithelial repair [19]. The primers for EP-1 and EP-4 were validated using a bladder smooth muscle and dorsal root ganglion RNA control, respectively, which showed the expression of each of the receptors. However, EP-2 and EP-3 receptors were readily detected (Fig. 4). EP-3 receptor expression increased significantly with time post-wounding when compared with the internal standard (Figs 4c and d), although this was not observed for EP-2 receptors (Figs 4a and b). A significant eight-fold increase in EP-3 receptor expression in wounded cells was confirmed by qRT-PCR analysis of cells following wounding and overnight incubation. There was no significant increase in EP-3 mRNA expression at the 6-h time-point, and no effect of wounding on EP-2 receptor expression at either time-point.

The effect of exogenously added factor Xa on wound repair

As fibrin formation mediated by extrinsic coagulation cascade proteases and protease-activated receptors appear to be involved in the epithelial repair response, we tested the effect of the exogenously added FXa on wound repair in a range of concentrations including those normally present in plasma. FXa significantly enhanced wound repair at all concentrations tested in a dose-dependent manner (Fig. 5).

Fig. 3. Effect of mechanical wounding, PAR-1 and PAR-2 peptide agonists and indomethacin on the concentration of (a) fibrinogen, (b) FXIII, (c) D-dimers in cell culture supernatants of 16HBE 14o⁻ cells 2 h postwounding confluent monolayers. C, confluent; W2, two wounds; W4, four wounds and W8, eight wounds. Data represent mean $\pm$ SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the no drug control.

The effect of wounding on interleukin-8 release in culture supernatants

As normal repair responses appear to be mediated via protease-activated receptors that in wounded cell cultures are activated by coagulation cascade proteases, and PAR

Table 2. The effect of scrambled control PAR-1 and PAR-2 peptide agonists on the release of fibrinogen and factor XIII, and the formation of D-dimers, 2 h after wounding (W) confluent (C) monolayers of 16 HBE cell monolayers

Coagulation factor	Condi-tion	Control peptide		
		No drug	PAR-1 (FTRIFD)	PAR-2 (LSIGKVD)
Fibrinogen (µg/mL)	C	0.67±0.44	0.22±0.01	0.76±0.64
	W2	3.18±2.29	0.91±0.03	2.22±0.24
	W4	8.41±1.39 [†]	3.55±0.21 ^{††}	4.33±0.32 ^{††}
	W8	21.23±1.33 ^{††}	5.32±0.48 ^{***††}	6.15±0.31 ^{***††}
Factor XIII (ng/mL)	C	4.49±1.96	2.18±0.08	2.92±0.25
	W2	17.00±1.52	7.30±0.22 ^{*††}	7.92±1.26 ^{*††}
	W4	22.30±2.40 [†]	10.03±0.75 ^{***††}	15.23±0.65 ^{††}
	W8	24.43±5.40 ^{††}	12.93±0.42 ^{***††}	18.39±0.59 ^{†††}
D-dimers (ng/mL)	C	2.33±0.27	4.19±1.32	2.94±0.60
	W2	6.54±0.72	6.46±1.17	3.62±0.84
	W4	10.42±2.03 ^{††}	8.34±0.95	7.36±0.84 [†]
	W8	16.21±1.10 ^{††}	10.89±0.75 ^{*†}	9.20±1.34 ^{***†††}

The degree of wounding is indicated as W2 (two wounds), W4 (four wounds) and W8 (eight wounds). The effect of wounding on coagulation factor concentration in culture supernatants was analysed by one-way ANOVA and Dunnett's *post hoc* test.

[†] $P < 0.05$, ^{††} $P < 0.01$ and ^{†††} $P < 0.001$. Data on the effect of peptides under each condition were analysed by two-way ANOVA and Bonferroni *post hoc* tests. Significant differences between the peptide-treated and no drug controls are indicated with asterisks; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Fig. 4. (a) Representative gel demonstrating the effect of time post-wounding on the mRNA expression of EP-2 in 16HBE 140⁻ cells. 2% w/v agarose gel electrophoretic separation of EP-2 amplicons generated by RT-PCR from the total RNA extracted from 16HBE 140⁻ cells at various time-points following mechanical wounding. Lane contents as follows: BP, 100 bp ladder; C, negative control (no RNA); BL, baseline (unwounded cells); 2 h, 2-h post-wound; 4 h, 4-h post-wound; 6 h, 6-h post-wound; 8 h, 8-h post-wound; 10 h, 10-h post-wound. (b) Effect of wounding on the mRNA levels of EP-2 in 16HBE 140⁻ cells. Data bars indicate the ratio of EP-2 to β -actin internal control. BL, baseline i.e. unwounded cell monolayers. Data represent mean±SEM of three independent experiments. (c) Representative gel demonstrating the effect of time post-wounding on the mRNA expression of EP-3 receptor in 16HBE 140⁻ cells. 2% (w/v) agarose gel electrophoretic separation of EP-3 amplicons generated by RT-PCR from the total RNA extracted from 16HBE 140⁻ cells at various time-points following mechanical wounding. Lane contents as follows: BP, 100 bp ladder; BL, baseline (unwounded cells); 2 h, 2-h post-wound; 4 h, 4-h post-wound; 6 h, 6-h post-wound; 8 h, 8-h post-wound; 10 h, 10-h post-wound. (d) Effect of wounding on the mRNA levels of EP-3 in 16HBE 140⁻ cells. Data bars indicate the ratio of EP-3 to β -actin internal control. BL, baseline i.e. unwounded cell monolayers. Data represent mean±SEM of four independent experiments. * $P < 0.05$ compared with BL.

activation is linked with inflammatory responses, we investigated the levels of the neutrophil chemoattractant IL-8 in supernatants from unwounded and wounded monolayer cultures 12-h post-wounding. IL-8 concentrations were 449±186 pg/mL in supernatants from unwounded monolayers and 1451±120 pg/mL, which is significantly ($P < 0.05$) higher in supernatants from wounded cultures ($n = 5$).

The effect of the neutrophil elastase on wound repair and coagulation factors

Neutrophil elastase has been reported to both activate [22] and disarm [13, 14] PARs, and to degrade coagulation factors including FVII [23], FX [24] and FXIIIa [25]. Therefore, it was of interest to investigate the effect of

Fig. 5. Effect of exogenous FXa on wound repair of 16HBE 140⁻ cell layers at 13 h. Data represent mean±SEM of three independent experiments. **P*<0.0005 and ***P*<0.0001 compared with no drug control.

Fig. 6. Effect of elastase on repair of 16HBE 140⁻ cell monolayers at 13 h. Data represent mean±SEM of three independent experiments. **P*<0.05 and ***P*<0.0001 compared with no elastase control.

this enzyme on epithelial repair. Neutrophil elastase significantly inhibited epithelial repair in a dose-dependent fashion in the concentration range of 0.01–0.5 U/mL (Fig. 6), indicating that neutrophil elastase did not activate PARs. In addition, immunohistochemical analysis indicated that neutrophil elastase (0.3 U/mL) did not cleave the N-terminal domains recognized by the antibodies, and therefore did not disarm either PAR-1 or PAR-2 (supporting information Figs S1 and S2).

The effect of neutrophil elastase on fibrinogen and FXIII levels in culture supernatants was investigated by immunoblot analysis. Neutrophil elastase reduced the levels of both fibrinogen and FXIII in culture supernatants harvested 2-h post-wounding in a concentration-dependent manner (Fig. 7). The significant increase in fibrinogen (Fig. 7a) and FXIII (Fig. 7b) seen following wounding in the absence of neutrophil elastase was significantly reduced in the presence of 0.1–0.5 U/mL neutrophil elastase, so that at 0.5 U/mL (0.34 µM), there was no longer any significant difference in the concentrations of coagulation factors between wounded and unwounded control cultures. Levels of coagulation factors in supernatants harvested 20 min after wounding were still significantly higher than the control unwounded cultures (not shown). The effect of neutrophil elastase was therefore both time and concentration dependent.

Fig. 7. (a) Effect of elastase on the concentration of fibrinogen in cell culture supernatants of 16HBE 140⁻ cells 2-h post-wounding. Control bars denote unwounded cells and wounded bars represent maximum scrape damage, W8. Data represent mean±SEM of three independent experiments. **P*<0.01 and ***P*<0.001 compared with control (unwounded) cells at each concentration of elastase. †*P*<0.01 compared with the no elastase control in the wounded cells. (b) Effect of elastase on the concentration of FXIII in cell culture supernatants of 16HBE 140⁻ cells at 2 h post-wounding. Control bars denote unwounded cells and wounded bars represent maximum scrape damage, W8. Data represent mean±SEM of three independent experiments. **P*<0.05 compared with control cells at each concentration of elastase. †*P*<0.01 compared with the no elastase control in the wounded cells.

Elastase in the concentration range of 0.01–0.5 U/mL had no effect on the distribution of LDH between the supernatants and cell lysates indicating that the inhibition of wound repair was not due to cytotoxicity induced by this enzyme.

Discussion

In addition to their role in fibrin formation, the role of the coagulation cascade proteases in lung inflammation, tissue remodelling and fibrosis in the airways has recently been reviewed [10, 12, 26]. Important roles for PAR-1 expressed on pulmonary fibroblasts in the development of fibrosis have been described, while the activation of epithelial PAR-2 results in both pro-inflammatory [12] and anti-inflammatory and cytoprotective effects [20]. We now report that PAR-1 and PAR-2 peptide agonists stimulate the rate of repair of mechanically wounded bronchial epithelial cell monolayers in culture and enhance the formation of a fibrin provisional matrix to support the repair process. Conversely, PAR-1- and PAR-

2-neutralizing antibodies inhibited the repair response. This indicates a role for *endogenous* proteases in the normal repair process, conceivably membrane-bound coagulation cascade proteases that we reported to be involved in epithelial repair [7], or soluble proteases released in response to wounding, such as MMP-1 or human airway trypsin (HAT) that also activate PAR-1 and PAR-2, respectively [27, 28].

Important roles for FXa, signalling through PAR-1 and PAR-2 receptors, in pro-inflammatory and pro-fibrotic responses following tissue injury have been described previously [26]. We demonstrated previously increased tissue factor (TF) and FVII expression in 16HBE 14o⁻ cells in response to wounding [7] and the activation of FXa in our model by the TF/FVIIa complex was indicated. Enhanced bronchial epithelial wound repair in response to the exogenously added FXa supports this previously unrecognized role for endogenous FXa. EGFR induces cell proliferation and migration and plays an important role in normal epithelial repair [29]. PAR-1 and PAR-2 transactivate epidermal growth factor receptor (EGFR) either by an activation of intracellular signalling pathways leading to src-dependent EGFR phosphorylation and/or the PAR-induced release of EGFR ligands [reviewed in [12]]. Thus, enhanced fibrin-dependent epithelial repair responses induced via the coagulation cascade proteases may prove to be mediated, indirectly, via EGFR activation.

The soluble peptides were chosen for their ability to act as selective agonists at the PAR-1 (TFRIFD-NH2) and PAR-2 (SLIGKVD-NH2) receptor [30, 31]. Reversal of the first two amino acids in the amino terminal of the PAR-1- and PAR-2-activating peptides SFLLRN and SLIGRL have consistently been shown to yield a functionally inactive peptide in many cells and tissues, and the FSLLRN and LSIKRL peptides are often used as control peptides [11, 18]. We therefore reversed the order of the first two amino acids in TFRIFD-NH2 and SLIGKVD-NH2 to provide control peptides. These control peptides had no stimulatory effect on wound closure by 16HBE cells (Fig. 1), or on the levels of fibrinogen, FXIIIa or D-dimers in control or wounded cultures (Table 2) indicating the specificity of the chosen agonist peptides.

Unexpectedly, the control peptides, FTRIFD-NH2 and LSIKVD-NH2, significantly decreased fibrin formation in wounded cell cultures. The inhibitory effect of the control peptides was only partial and the release of fibrinogen and FXIIIa, and the formation of fibrin was still significantly increased in wounded cell cultures compared with confluent monolayers (Table 2). A major role for the S³⁷L³⁸ dipeptide sequence in PAR-2 activation by SLIGRL has been reported [32] and a reversal of this sequence generates a peptide that inhibits the ability of exogenously added trypsin and the newly formed tethered ligand, but not the soluble activating peptide, to activate PAR-2 [33]. Similarly, an essential role for the initial dipeptide sequence and

particularly phenylalanine, which must be preceded by another amino acid in the TFRIFD sequence, has been indicated [34]. Modification of the human PAR-1 peptide by the deletion of the first amino acid produced a thrombin antagonist. The control peptides used in our study, with the first two amino acids reversed, apparently acted as inhibitors or antagonists of endogenous PAR-1- and PAR-2-activating proteases involved in fibrin formation.

Importantly, the control peptides did not affect the rate of epithelial repair (Fig. 1). We demonstrated previously an essential role for tissue factor and proteases of the coagulation cascade in fibrin formation and wound repair [7]. We also reported that when tissue factor-mediated activation of coagulation cascade proteases was inhibited, fibrinogen continued to accumulate in the culture supernatant of wounded cell monolayers [7]. Together with the present findings, regarding the stimulatory effects of PAR activation on fibrinogen and FXIIIa release and D-dimer formation, our findings are indicative of an additional effect of soluble proteases such as matrix metalloproteases and human airway trypsin, released in response to wounding and able to activate PAR-1 and PAR-2, respectively. Further experiments are needed to investigate whether the scrambled peptides limit PAR activation by endogenous soluble and/or membrane-bound proteases in our model, or act as PAR antagonists.

Immunohistochemical analysis revealed that PARs 1–4 are expressed in the normal bronchial epithelium, and RT-PCR analysis of primary cells and epithelial cell lines in culture has confirmed this [15, 16]. PAR expression is upregulated by a number of different stimuli and a modulation of receptor expression regulates PAR activity [12]. PAR-1 and PAR-2 expression are up-regulated during the development of respiratory diseases such as pulmonary fibrosis, asthma and bronchitis, in which they play a role in the development of disease pathology. Notably, PAR-1 is strongly associated with the development of tissue fibrosis [10] and PAR-2 with inflammatory airways diseases [12]. The 16HBE 14o⁻ cell line has been shown previously to express PAR-1 [21] and PAR-2 [21, 14], which we have confirmed in this study for cells growing in serum-free conditions. It was reported previously that continued exposure to PAR-activating peptides enhances PAR expression in A549 epithelial cells [12]. We considered the possibility that the proposed continued stimulation of PARs by *endogenous* coagulation cascade proteases following wounding in our model may relate to a small sub-population of cells at the wound edge that did not lead to a detectable overall increase in PAR-1 or PAR-2 expression. However, there is little evidence for an increased expression of PAR-1 and PAR-2 receptors with wounding or on exposure to high concentrations of PAR-activating peptides at the mRNA or protein level.

Initially, we were surprised to find no effect on the rate of epithelial repair on the addition of PGE2 in the

concentration range shown previously by Savla et al. [19] to enhance the repair of mechanically wounded 16HBE 140⁻ cell layers. In the study by Savla and colleagues (2001), cells were grown in a medium containing 10% fetal bovine serum, and the effect of PGE2 added in a medium containing 10% fetal bovine serum was mediated by EP-1 and EP-4 receptors. However, in our serum-free conditions, EP-1 and EP-4 were not expressed, either at baseline or following wounding, although the expression of the EP-2 and EP-3 receptors was readily detected. Subsequent experiments showed that EP-1 and EP-4 were also not expressed at baseline or following wounding, in the presence of serum (results not shown). Because the primers for EP-1 and EP-4 were validated using a bladder smooth muscle and dorsal root ganglion RNA control, respectively, it appears that the 16HBE 140⁻ cell line expresses only EP-2 and EP-3 under our culture conditions. Additionally, the study by Savla et al. [19], used the non-specific receptor antagonists AH6809 and AH23848 to indicate the involvement of EP-1 and EP-4 receptors, respectively, in epithelial repair. However, AH6809 is an antagonist equally at EP1, EP2 and EP3 receptors, as well as DP and TP receptors, and AH23848 is a more potent antagonist of EP-3 than EP-4 receptors, and is also an antagonist at DP and TP receptors [35]. Therefore, considering the selectivity and affinity of the EP receptor agonists and antagonists used by Savla et al. [19], it is possible that EP-2 or EP-3 receptors mediated epithelial repair responses in that study.

We reported previously very low levels of PGE2 that did not increase with wounding in our serum-free model of bronchial epithelial repair [7]. Serum-free conditions are known to limit PGE2 production [36, 37], and it was therefore not surprising to observe no effect of indomethacin on the rate of wound repair or on coagulation factor release in response to wounding alone. However, a significant inhibitory effect of indomethacin on fibrin formation, measured as D-dimer concentration, indicates that PGE2 or other prostanoid was produced in response to the additive effect of both a mechanical wound and the PAR-2-activating peptide, but not in control unwounded cell cultures. PAR-1- and PAR-2-activating peptides at 400 µM concentrations have been shown previously to stimulate bronchial epithelial cell lines to synthesize PGE2 [18], but at levels too low to stimulate repair [19], and the effect of a PAR-2 agonist was greater than a PAR-1-activating peptide [18]. PAR-activating peptides have relatively low potency and since the peptides are susceptible to aminopeptidases, their degradation would be enhanced in serum-free conditions. It has been suggested previously that PGE2 is only produced at effective concentrations when PAR-2 is activated strongly, for example by high concentrations of activating peptides that maintain its concentration in the cell environment and cause sustained activation [13]. Thus, if the peptides were

present at suboptimal concentrations, a second, proteolytic, PAR-2-activating stimulus from wounded cell layers, such as FXa or human airway trypsin may generate sufficient PGE2 to influence fibrin formation. However, analysis of culture supernatants under all conditions of wounding and in the presence the PAR-activating peptides found only pg/mL levels of PGE2 and PGE2 metabolites (data not shown). Also, as exogenously added PGE2, alone, did not affect the rate of wound repair or coagulation factor expression in wounded and unwounded cultures, an effect of indomethacin on the synthesis of other prostanoids, or other cyclooxygenase-independent effects [38], is likely.

In evolutionary terms, the role of coagulation enzymes is related to the formation of fibrin at wound sites and the induction of an inflammatory response, which developed as a rapid response system to detect and clear microbes that have breached epithelial barriers, namely the exclusion of invading pathogens by the innate immune system [39, 40]. PARs play a crucial role in the cross-talk between coagulation and inflammation [41, 26]. Activation of the coagulation cascade not only leads to fibrin formation but, also via PAR-1 and PAR-2 activation, the release of pro-inflammatory mediators that stimulate tissue factor expression, and the activity of the extrinsic coagulation cascade leading to persistent fibrin formation [10]. PAR-1 and PAR-2 activation were reported previously to stimulate IL-8 release from A549, BEAS-2B and primary bronchial epithelial cells, although PAR-2 agonist peptides were more potent and more effective than PAR-1-activating peptides [18]. Others, using the same peptide agonists as those used in our study, showed that IL-8 release from 16HBE 140⁻ cells was stimulated by PAR-2 but not PAR-1 agonists [21]. Post-transcriptional upregulation of IL-8 release from A549 cells through PAR-2-dependent transactivation of EGF receptors has also been described recently [42]. As PGE2 exerts a positive transcriptional regulation of IL-8 synthesis in a number of cells including fibroblasts, T-cells, colonic and pulmonary epithelial cells [43] and as our serum-free conditions limit PGE2 synthesis, we investigated whether wounding alone induced IL-8 synthesis under our assay conditions. The increased synthesis and release of IL-8 in our wounded cultures, in the absence of exogenously added PAR-activating proteases or peptides, is indicative of either PAR-1 and/or PAR-2 activation by *endogenous* proteases, either coagulation cascade proteases or others released from wounded cells.

IL-8 is a potent neutrophil chemoattractant and the normal physiologic role of neutrophils recruited to wound sites is to aid in the repair process by clearing foreign particles and microbes, tissue debridement and wound cleaning [44]. However, in various pathological conditions neutrophil elastase is released from activated cells into the extracellular matrix where it has a number of

well-recognized roles in both tissue injury (matrix destruction) and repair (matrix accumulation) [45–47]. Studies have shown that elastase activates PAR-1 in human lung epithelial cells to induce apoptosis [22] but disarms PAR-2 in lung epithelial cells [13, 14]. These effects appear to be specific for the epithelial cell type, as elastase disarms PAR-1, but activates PAR-2, in non-epithelial cells [13]. We found that neutrophil elastase significantly inhibited epithelial repair, which may be the result of the degradation of coagulation factors, as neutrophil elastase did not apparently disarm PAR-2 receptors on epithelial cells. FXIIIa cross-links and stabilizes fibrin and plays a role in angiogenesis and tissue repair [48]. We demonstrated previously an essential role for FXIIIa in epithelial repair [7] and its degradation by neutrophil elastase has been described previously [25]. Thus it seems likely that degradation of FXIIIa, fibrinogen or even cross-linked fibrin [49] by neutrophil elastase is at least partly responsible for inhibition of epithelial repair.

The airway epithelium is central to the pathogenesis of asthma [50]. An inherent fragility in the bronchial epithelium resulting from defective tight junctions is a feature of the asthmatic airway [27], which increases the risk of asthma exacerbation due to environmental factors [51]. In addition, a lower level of anti-protease expression in the asthmatic airway epithelium compared with normal has been suggested to render the airway epithelium in asthmatics more susceptible to damage via allergens with proteolytic activity [52]. Our findings may be relevant to the airway epithelium in severe asthma, which is dominated by a neutrophilic inflammatory response [53]. As we have shown in this study, neutrophil elastase not only damages the airway epithelium [46] but also inhibits the repair response. Significant airway epithelial damage is associated with plasma exudation [54] and since tissue factor-bearing cells are believed to be involved only in the initiation of clot formation, the growth of the clot is dependent on the propagation of coagulation to bulk plasma [55]. Thus, while plasma exudation is normally considered a defensive repair response, plasma exudation may become a pathophysiological event supporting excessive fibrin formation in severe asthma [56, 57], as we demonstrated recently [58], as well as acute inflammatory and chronic interstitial lung diseases [59].

Acknowledgements

This study was funded by Pfizer Global R&D. The authors have declared no conflict of interest.

References

- 1 Bals R, Hiemstra PS. Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. *Eur Respir J* 2004; 23:327–33.

- 2 Kato A, Schleimer RP. Beyond inflammation: airway epithelial cells are at the interface of innate and adaptive immunity. *Curr Opin Immunol* 2007; 19:711–20.
- 3 Erjefalt JS, Korsgren M, Nilsson MC, Sundler F, Persson CGA. Association between inflammation and epithelial damage–restitution processes in allergic airways in vivo. *Clin Exp Allergy* 1997; 27:1344–55.
- 4 Gershwin LJ. Effects of allergenic extracts on airway epithelium. *Curr Allergy Asthma Rep* 2007; 7:357–62.
- 5 Reed CE. Inflammatory effect of environmental proteases on airway mucosa. *Curr Allergy Asthma Rep* 2007; 7:368–74.
- 6 Erjefalt JS, Erjefalt I, Sundler F, Persson CGA. Microcirculation-derived factors in airway epithelial repair in vivo. *Microvasc Res* 1994; 48:161–78.
- 7 Perrio MJ, Ewen D, Trevethick MA, Salmon GP, Shute JK. Fibrin formation by wounded bronchial epithelial cell layers in vitro is essential for normal epithelial repair and independent of plasma proteins. *Clin Exp Allergy* 2007; 37:1688–700.
- 8 Macfarlane SR, Seatter MJ, Kanke T, Hunter GD, Plevin R. Proteinase-activated receptors. *Pharm Rev* 2001; 53:245.
- 9 De Campo BA, Henry PJ. Protease-activated receptors (PARs) are partly pro-inflammatory and partly anti-inflammatory: will PAR agonists or antagonists participate in future drug therapies? *Curr Drug Targets* 2006; 7:629–37.
- 10 Chambers R. Procoagulant signalling mechanisms in lung inflammation and fibrosis: novel opportunities for pharmacological intervention? *Br J Pharmacol* 2008; 153:S367–78.
- 11 Lan RS, Stewart GA, Henry PJ. Role of protease-activated receptors in airway function: a target for therapeutic intervention? *Pharmacol Therapeut* 2002; 95:239–57.
- 12 Sokolova E, Reiser G. A novel therapeutic target in various lung diseases: airway proteases and protease-activated receptors. *Pharmacol Therapeut* 2007; 115:70–83.
- 13 Chignard M, Pidard D. Neutrophil and pathogen proteinases versus proteinase-activated receptor-2 lung epithelial cells. More terminators than activators. *Am J Respir Cell Mol Biol* 2006; 34:394–8.
- 14 Dulon S, Cande C, Bunnett NW, Hollenberg MD, Chignard M, Pidard D. Proteinase-activated receptor-2 and human lung epithelial cells. Disarming by neutrophil serine proteases. *Am J Respir Cell Mol Biol* 2003; 28:339–46.
- 15 Knight DA, Lim S, Scaffidi AK *et al.* Protease-activated receptors in human airways: upregulation of PAR-2 in respiratory epithelium from patients with asthma. *J Allergy Clin Immunol* 2001; 108:797–803.
- 16 Cocks TM, Fong B, Chow JM *et al.* A protective role for protease-activated receptors in the airways. *Nature* 1999; 398:156–60.
- 17 Cocks TM, Moffatt JD. Protease-activated receptor-2 (PAR2) in the airways. *Pulm Pharm Therapeut* 2001; 14:183–91.
- 18 Asokanathan N, Graham PT, Fink J *et al.* Activation of protease-activated receptor (PAR)-1, PAR-2, and PAR-4 stimulates IL-6, IL-8, and prostaglandin E2 release from human respiratory epithelial cells. *J Immunol* 2002; 168:3577–85.
- 19 Savla U, Appel HJ, Sporn PHS, Waters CM. Prostaglandin E2 regulates wound closure in airway epithelium. *Am J Physiol Lung Cell Mol Physiol* 2001; 280:L421–31.
- 20 Henry PJ. The protease-activated receptor2 (PAR2)-prostaglandin E2-prostanoid EP receptor axis: a potential bronchoprotective unit in the respiratory tract? *Eur J Pharmacol* 2006; 533:156–70.

- 21 Page K, Strunk VS, Hershenon MB. Cockroach proteases increase IL-8 expression in human bronchial epithelial cells via activation of protease-activated receptor (PAR)-2 and extracellular-signal-regulated kinase. *J Allergy Clin Immunol* 2003; 112:1112–8.
- 22 Suzuki T, Moraes TJ, Vachon E *et al*. Proteinase-activated receptor-1 mediates elastase-induced apoptosis of human lung epithelial cells. *Am J Respir Cell Mol Biol* 2005; 33:231–47.
- 23 Anderssen T, Halvorsen H, Bajaj SP, Osterud B. Human leukocyte elastase and cathepsin G inactivate factor VII by limited proteolysis. *Thromb Haemostasis* 1993; 70:414–7.
- 24 Turkington PT. Degradation of human factor X by human polymorphonuclear leucocyte cathepsin G and elastase. *Haemostasis* 1991; 21:111–6.
- 25 Bagoly Z, Fazakas F, Komaromi I, Haramura G, Toth E, Muszbek L. Cleavage of factor XIII by human neutrophil elastase results in a novel active truncated form of factor XIIIa subunit. *Thromb Haemostasis* 2008; 99:668–74.
- 26 Krupiczkoj MA, Scotton CJ, Chambers RC. Coagulation signalling following tissue injury: focus on the role of factor Xa. *Int J Biochem Cell Biol* 2008; 40:1228–37.
- 27 Blackburn JS, Brinckerhoff CE. Matrix metalloproteinase-1 and thrombin differentially activate gene expression in endothelial cells via PAR-1 and promote angiogenesis. *Am J Pathol* 2008; 173:1736–46.
- 28 Chokki M, Eguchi H, Hamamura I, Mitsuhashi H, Kamimura T. Human airway trypsin-like protease induces amphiregulin release through a mechanism involving protease-activated receptor-2 mediated ERK activation and TNF α -converting enzyme activity in airway epithelial cells. *FEBS J* 2005; 272:6387–99.
- 29 Holgate ST. Epithelium dysfunction in asthma. *J Allergy Clin Immunol* 2007; 120:1233–44.
- 30 Blackhart BD, Emilsson K, Nguyen D *et al*. Ligand cross-reactivity within the protease-activated receptor family. *J Biol Chem* 1996; 271:16466–71.
- 31 Hollenberg MD, Saifeddine M, Al-Ani B, Kawabata A. Proteinase-activated receptors: structural requirements for activity, receptor cross-reactivity, and receptor selectivity of receptor-activating peptides. *Can J Physiol Pharmacol* 1997; 75:832–41.
- 32 Al-Ani B, Hansen KK, Hollenberg MD. Proteinase-activated receptor-2: key role of amino-terminal dipeptide residues of the tethered ligand for receptor activation. *Mol Pharmacol* 2004; 65:149–56.
- 33 Al-Ani B, Saifeddine M, Wijesuriya J, Hollenberg MD. Modified proteinase-activated receptor-1 and -2 derived peptides inhibit proteinase-activated receptor-2 activation by trypsin. *J Pharmacol Exp Ther* 2002; 300:702–8.
- 34 Vassallo RR, Kieber-Emmons T, Cichowski K, Brass LF. Structure–function relationships in the activation of platelet thrombin receptors by receptor-derived peptides. *J Biol Chem* 1992; 267:6081–5.
- 35 Abramovitz M, Adam M, Boie Y *et al*. The utilisation of recombinant prostanoid receptors to determine the affinities and selectivities of prostaglandins and related analogs. *Biochim Biophys Acta* 2000; 1483:285–93.
- 36 Alpert SE, Walenga RW, Kramer CM, Silski CL, Davies PB. Tracheal epithelial cell fatty acid composition modulates prostaglandin E2 and cAMP production. *Am J Physiol* 1992; 262:L192–7.
- 37 Dewitt DL, Meade EA. Serum and glucocorticoid regulation of gene transcription and expression of the prostaglandin H synthase-1 and prostaglandin H synthase-2 isozymes. *Arch Biochem Biophys* 1993; 306:94–102.
- 38 Tegeder I, Pfeilschifter J, Geisslinger G. Cyclooxygenase-independent actions of cyclooxygenase inhibitors. *FASEB J* 2001; 15:2057–72.
- 39 Opal SM. Interactions between coagulation and inflammation. *Scand J Infect Dis* 2003; 35:545–54.
- 40 Spronk HMH, Govers-Riemsag JWP, ten Cate H. The blood coagulation system as a molecular machine. *BioEssays* 2003; 25:1220–8.
- 41 Dugina TN, Kiseleva EV, Chistow IV, Umarova BA, Strukova SM. Receptors of the PAR family as a link between blood coagulation and inflammation. *Biochemistry (Moscow)* 2002; 67:65–74.
- 42 Moriyuki K, Nagataki M, Sekiguchi F, Nishikawa H, Kawabata A. Signal transduction for formation/release of interleukin-8 caused by a PAR-2 activating peptide in human lung epithelial cells. *Reg Peptides* 2008; 145:42–8.
- 43 Peng H, Chen P, Cai Y *et al*. Endothelin-1 increases expression of cyclooxygenase-2 and production of interleukin-8 in human pulmonary epithelial cells. *Peptides* 2008; 29:419–24.
- 44 Tsirogiani AK, Moutsopoulos NM, Moutsopoulos HM. Wound healing: immunological aspects. *Injury* 2006; 37:S5–12.
- 45 Chua F, Laurent GJ. Neutrophil elastase. Mediator of extracellular matrix destruction and accumulation. *Proc Am Thorac Soc* 2006; 3:424–7.
- 46 Lungarella G, Cavarra E, Lucatelli M, Martorana PA. The dual role of neutrophil elastase in lung destruction and repair. *Int J Biochem Cell Biol* 2008; 40:1287–96.
- 47 Liu C-Y, Liu Y-H, Lin S-M *et al*. Apoptotic neutrophils undergoing secondary necrosis induce human lung epithelial cell detachment. *J Biomed Sci* 2003; 10:746–56.
- 48 Inbal A, Dardik R. Role of coagulation factor XIII (FXIII) in angiogenesis and tissue repair. *J Pathophysiol Haemost Thromb* 2006; 35:162–5.
- 49 Travis J. Structure, function and control of neutrophil proteinases. *Am J Med* 1988; 84:37–42.
- 50 Holgate ST. The airway epithelium is central to the pathogenesis of asthma. *Allergol Int* 2008; 57:1–10.
- 51 D'Amato G, Liccardi G, D'Amato M, Holgate S. Environmental risk factors and allergic bronchial asthma. *Clin Exp Allergy* 2005; 35:1113–24.
- 52 Mattoli S. Allergen-induced generation of mediators in the mucosa. *Env Health Perspect* 2001; 109:553–7.
- 53 Holgate ST, Polosa R. The mechanisms, diagnosis, and management of severe asthma in adults. *The Lancet* 2006; 368:780–93.
- 54 Persson CGA. Airway epithelium and microcirculation. *Eur Respir Rev* 1994; 4:352–62.
- 55 Ovanesov MV, Ananyeva NM, Panteleev MA, Ataullakhanov FI, Saenko EL. Initiation and propagation of coagulation from tissue-factor-bearing cell monolayers to plasma: initiator cells do not regulate spatial growth. *J Thromb Haemostasis* 2005; 3:321–31.
- 56 Matthay MA, Clements JA. Coagulation-dependent mechanisms and asthma. *J Clin Invest* 2004; 114:20–3.
- 57 Wagers SS, Norton RJ, Rinaldi LM, Bates JHT, Sobel BE, Irvin CG. Extravascular fibrin, plasminogen activator, plasminogen activator inhibitors, and airway hyperresponsiveness. *J Clin Invest* 2004; 114:104–11.

- 58 Brims FJ, Chauhan AJ, Higgins B, Shute JK. Coagulation factors in the airways in moderate and severe asthma and the effect of inhaled steroids. *Thorax* 2009; **64**:1037–43.
- 59 Wygracka M, Jablonska E, Guenther A, Preissner KT, Markart P. Current view on alveolar coagulation and fibrinolysis in acute and chronic interstitial lung diseases. *Thromb Haemostasis* 2008; **99**:494–501.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Immunostaining of PAR-1 in 16HBE 14o[−] cell layers. a; no primary antibody negative control. LHS –

fluorescent image, RHS – DIC image, b; confluent cell layer, c; cell layer 2 h post wounding, d; cell layer treated with neutrophil elastase (0.3 U/mL, 200 nM) for 2.5 h, e; cell layer treated with neutrophil elastase (0.3 U/mL, 200 nM) 2 h post wounding.

Figure S2. Immunostaining of PAR-2 in 16HBE 14o[−] cell layers. a; confluent cell layer, b; cell layer 2 hours post wounding, c; cell layer treated with neutrophil elastase (0.3 U/mL, 200 nM) for 2.5 h, d & e; cell layer treated with neutrophil elastase (0.3 U/mL, 200 nM) 2 h post wounding.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.