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Fibrin formation by wounded bronchial epithelial cell layers *in vitro* is essential for normal epithelial repair and independent of plasma proteins

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Clinical and Experimental Allergy

Summary

Background The bronchial epithelium is in contact with, and continually damaged by, the environment. Animal models have indicated that normal epithelial repair is rapid and supported by the formation of a provisional fibrin matrix that is exclusively plasma-derived. **Objectives** Our objectives were to demonstrate the ability of normal human bronchial epithelial (NHBE) cells to produce coagulation cascade proteins and form fibrin in response to damage, *independently* of plasma proteins, and to show that formation of a cross-linked fibrin matrix is essential for normal epithelial repair *in vitro*.

Methods Primary NHBE cells and cells of the 16HBE 140- bronchial epithelial cell line were grown and maintained *in vitro* prior to mechanical wounding of confluent monolayers in serum-free media. Tissue factor (TF) and factor XIII (FXIII) were visualized on 16HBE 140- monolayers using immunohistochemistry. The time-dependent expression of TF, factor VII (FVII), factor X (FX), fibrinogen, soluble fibrin, FXIII subunit A (FXIIIA) and D-dimers following wounding of confluent 16HBE 140- monolayers was investigated using immunoassays. TF and FVII expression at the mRNA level was investigated by RT-PCR. The role of coagulation cascade proteins in the repair response of NHBE and 16HBE 140- monolayers was investigated using neutralizing antibodies.

Results Active TF was constitutively expressed in 16HBE 140- cells. Levels of FVII, FX, fibrinogen, soluble fibrin, FXIIIA and D-dimers in culture supernatants increased rapidly and were maximal 20 min after wounding the monolayers. Expression of TF and FVII mRNA was significantly increased 10 and 4 h, respectively, after wounding. Neutralizing antibodies to TF, fibrinogen and FXIIIA significantly inhibited repair of NHBE and 16HBE 140- cell layers.

Conclusions The bronchial epithelium has the potential to respond rapidly to mechanical damage by forming a cross-linked fibrin matrix that is essential for normal epithelial repair, *independently* of plasma proteins.

Keywords asthma, bronchial epithelium, coagulation cascade, fibrin matrix, repair

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Introduction

The normal bronchial epithelium protects the underlying tissue from the effects of inhaled particles, infectious agents, allergens, chemicals and pollutants. It is therefore important that the barrier properties of this epithelium are maintained and there is evidence from animal studies *in vivo* that airway epithelial repair following damage is both rapid and efficient [1]. Repair occurs through five major steps, namely restitution or migration of epithelial cells to cover the denuded area, proliferation and regulated dif-

ferentiation of epithelial cells, cell death during resolution of epithelial hyperplasia, restoration of the normal proportions of cell types and adaptive changes in the differentiation state of repairing epithelial cells [2]. Preceding these events and within minutes of epithelial damage, exudation of unfiltered plasma generates a fibrin-rich gel in which epithelial restitution proceeds rapidly [1, 3].

Evolutionary links have indicated that the role of coagulation enzymes is related to the function of coagulation in the innate immune system, which developed as a rapid response system to detect and clear microbes that

have breached epithelial barriers, namely immune exclusion of invading pathogens by the formation of fibrin at wound sites and the induction of an inflammatory response [4, 5]. An adhesive fibrin network was demonstrated to stimulate the rate of re-epithelialization of skin wounds [6]. However, much less is known about repair of the relatively less accessible bronchial epithelium. Unlike other epithelial surfaces such as the skin and the gut, normal airway epithelia have very slow turnover [7], and are replaced every 3–4 months [8], except following injury. Injury and loss of normal epithelial integrity is induced by allergens [9], respiratory viruses, particulates and oxidants [10]. In asthmatics, the bronchial epithelium is rendered more susceptible to these insults by inherent defects in innate defence and repair mechanisms, reacting as a chronic wound in which delayed healing maintains the inflammatory response [11]. Chronic inflammation is associated with plasma exudation in the asthmatic airway [12] that may contribute to repair responses [3]. Nevertheless, disruption of epithelial integrity with shedding of columnar epithelial cells and, to a lesser degree, basal cells with denudation of the epithelial basement membrane remains a characteristic of the asthmatic airway in adults and children [13, 14].

In the lung, fibrin deposition in response to injury may be part of normal repair and restoration of lung function. However, plasma exudation is a two-edged sword, and the excessive extravascular fibrin deposition that is characteristic of adult respiratory distress syndrome [15] and acute lung injury [16] is a target for anti-coagulation therapy. Fibrin was also detected on the luminal surface of distal airways of a patient who died in status asthmaticus and, in animal models, was associated with bronchial hyperresponsiveness [17].

Tissue factor (TF) is the primary initiator of the extrinsic coagulation cascade at extravascular sites. In normal healthy tissues, TF is expressed by the bronchial mucosa and alveolar epithelial cells, but is not expressed by vascular endothelial cells or circulating blood cells [18]. At these extravascular sites, TF is believed to provide a 'haemostatic envelope' that upon disruption of vascular integrity and exposure to plasma proteins initiates activation of coagulation through formation of the TF-factor VII (FVII) complex [19]. Binding to TF induces activation of FVII and subsequently proteolytic activation of factor X (FX), leading ultimately to thrombin formation and the cleavage of fibrinogen to fibrin [20]. The fibrin clot is cross-linked and stabilized by thrombin-activated factor XIII (FXIII) [21], and finally removed by plasmin-mediated fibrinolysis.

In view of the constant interaction of the bronchial epithelium with potentially damaging agents in the environment, an ongoing repair process in the healthy airway is assumed. We hypothesize that, in normal tissue, bronchial epithelial cells are able to support a

fibrin-dependent repair process in the absence of plasma exudation. This would require the local synthesis of coagulation factors and fibrinogen, proteins that are synthesized mainly in the liver. Alveolar epithelial cells in culture have previously been shown to generate their own matrix proteins, including fibrinogen [22]. We therefore sought evidence that bronchial epithelial cells could produce a fibrin provisional matrix in the absence of plasma and that this was essential for normal bronchial epithelial repair.

Methods

Cell culture

16HBE 140- cells were cultured in full minimal essential media (MEM; GibcoBRL, Paisley, UK) containing 10% heat-inactivated fetal calf serum (FCS; GibcoBRL), 2 mM L-glutamine (GibcoBRL) and 1% antibiotic/antimycotic (GibcoBRL, 100 U penicillin G, 100 µg streptomycin and 250 ng amphotericin B/mL) in 75 cm³ tissue culture-grade flasks at 37 °C in 5% CO₂. At 80–90% confluence the cells were passaged using 0.05% trypsin–0.53 mM ethylenediamine tetraacetic acid (EDTA) (GibcoBRL). After re-suspending in 1 mL full media the cells were counted and seeded at 100 000 cells per well in a 24-well plate, and cultured for 1–2 days. At confluence the cells were washed and then serum-starved overnight in MEM containing 2 mM L-glutamine, 1% antibiotic/antimycotic and 1% insulin-transferrin-selenium (ITS; Sigma, Poole, Dorset, UK). Prior to scrape damage the cells were washed in phosphate-buffered saline (PBS) and 250 µL fresh MEM-ITS was added to each well.

Normal human bronchial epithelial (NHBE) cells in bronchial epithelial growth medium (BEGM) with retinoic acid (< 1 µg/mL) were purchased from Cambrex BioScience (Wokingham, UK). Cells were seeded at 100 000 cells per well into 24-well plates pre-coated with collagen IV at 10 µg/cm² and cultured in fully supplemented BEGM (BulletKit[®]: Cambrex BioScience), and 1% (v/v) heat-inactivated FBS at 37 °C in a 5% CO₂ incubator for 5 days until fully confluent. NHBE cells were used with a maximum of three passages. At confluence, cells were washed and quiesced overnight in 250 µL of serum-free BEGM-ITS [basal BEGM containing 2 mM L-glutamine and antibiotic/antimycotic (100 U gentamicin/250 ng amphotericin B/mL), supplemented with 1% (v/v) ITS solution]. Immediately prior to each experiment, cells were washed with PBS and 250 µL fresh BEGM-ITS was added to each well.

Mechanical wounding

Scrape damage was applied in a cross-hatch pattern of two, four or eight scrapes using a P200 yellow pipette tip to produce a wound 0.8 mm wide. Damaged cultures were

incubated for either 20 min or 2 h. Supernatants were removed, and the cell debris was cleared and stored in Eppendorf tubes at -80°C . The cells were washed in PBS, 250 μL ice-cold lysis buffer [10 mM Tris-HCl pH 7.4, 0.1 mM CaCl_2 , 0.1% bovine serum albumin (BSA) and 1% Triton-X-100 (v/v; Sigma)] was added, and plates were stored at -80°C .

Tissue factor and factor XIII subunit A immunostaining

16HBE 14o- cells were cultured in Microtek eight-well chamber slides (Nunc, Naperville, IL, USA) at 50 000 cells per well for 48 h. At confluence, cells were washed with 500 μL of PBS per well and quiesced for 16 h in MEM-ITS. Prior to each experiment, MEM-ITS was refreshed. Cells were mechanically wounded with a P2 Gilson pipette tip to produce a 0.5 mm wound in a cross-hatch manner (+) and returned to the incubator at 37°C for a time course of 30 min, 1 and 2 h. Supernatants were removed and cells were washed with 500 μL of PBS per well. Cells were fixed by adding 500 μL per well of 4% (w/v) paraformaldehyde in PBS for 30 min at room temperature and then washed three times by adding 500 μL of PBS per well and permeabilized by adding 500 μL per well of ice-cold methanol (100%), for 5 min at -20°C . Cells were washed three times by adding 500 μL of PBS per well and incubated for 16 h in PBS containing 1% (w/v) BSA at 4°C to block non-specific binding sites. Cells were incubated with primary mouse anti-human TF antibody (American Diagnostica, Stamford, CT, USA) or sheep anti-human FXIII subunit A (FXIIIa) (Affinity Biologicals, Ontario, Canada) at 5 $\mu\text{g}/\text{mL}$ in PBS-1% BSA for 1 h at room temperature. Cells were washed five times for 5 min each with 500 μL of PBS-0.02% (v/v) Tween 20 per well and incubated with secondary antibody (goat anti-mouse Alexa Fluor[®] 488 F(ab')₂ fragment or donkey anti-sheep Alexa Fluor[®] 594, Cambridge Bioscience, Cambridge, UK) at 5 $\mu\text{g}/\text{mL}$ in PBS-1% BSA for 1 h at room temperature. Cells were washed five times for 5 min each with 500 μL of 1 $\times$ PBS-0.02% (v/v) Tween 20 and the slide was mounted using Vectashield mounting medium (Vector Labs, Peterborough, UK). Cells were viewed using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany).

Tissue factor enzyme-linked immunosorbent assay

TF was measured in cell lysates by ELISA using paired antibodies from Affinity Biologicals according to the manufacturer's instructions. Standards were prepared from recombinant TF (American Diagnostica) diluted in the range of 50, 25, 12.5, 6.25, 3.13 and 1.56 ng/mL with sample diluent.

Immunoblotting for coagulation factors

Supernatants (3 μL) and standards (3 μL) normal pooled plasma (Alpha Laboratories, Eastleigh, UK) (diluted in the

range between 0.1% and 10%) were applied onto a 0.4 μm nitrocellulose membrane (BioRad, Hertfordshire, UK) using a 96-well grid for guidance. Samples were air dried and subsequently blocked overnight in PBS-2% Tween 20 (v/v) at 4°C , and then washed three times for 10 min in PBS-0.05% Tween 20 (v/v), while shaking. Peroxidase-conjugated IgG antibodies (2 mg/mL, Affinity Biologicals) were diluted in PBS-2% Tween 20 and applied to the surface of the blots for 1 h at room temperature: sheep anti-human FVII (1 : 1000), sheep anti-human FXIIIa (1 : 20 000), sheep anti-human Fibrinopeptide A (1 : 10 000) to detect fibrinogen, and goat anti-human FX (1 : 10 000). The blots were washed five times for 10 min with PBS-0.05% Tween 20. Chemiluminescent detection was carried out with Pierce SuperSignal West Pico Substrate (Pierce, Warriner, UK) and Super RX Fugifilm (Dusseldorf, Germany) were exposed to the membranes and developed using a Kodak X-ray film developer. The intensity of the dots was assessed by scanning densitometry using Quantiscan software (Biosoft, Cambridge, UK); the results were then aligned to a standard curve and adjusted based on reference values found in normal pooled plasma [Fibrinogen: 3000 $\mu\text{g}/\text{mL}$; FVII: 0.5 $\mu\text{g}/\text{mL}$; FX: 8 $\mu\text{g}/\text{mL}$; FXIII: 10 $\mu\text{g}/\text{mL}$; Ref: Chromogenix (Milan, Italy) haemostasis products catalogue, based on a review of current literature].

The antibody specificity was checked against normal plasma and coagulation factor-depleted plasma. Additionally, non-specific binding was tested against the range of albumin concentrations (0.05–5.0 mg/mL) found in 0.1–10% plasma. None of the antibodies bound non-specifically to any concentration of albumin.

D-dimer immunoblots: Supernatants (20 μL) and standards (20 μL) of high-molecular-weight D-dimer control (American Diagnostica; diluted in the range between 49.2 and 0.3 ng/mL) were applied under vacuum to a hybridization chamber containing 0.4 μm nitrocellulose membrane (BioRad) placed upon filter paper that had been previously soaked in PBS. Samples were air dried and subsequently blocked overnight in PBS-2% Tween 20 (v/v) at 4°C , and then washed three times for 10 min in PBS-0.05% Tween 20. Mouse anti-human D-dimer MAb (0.5 mg/mL, American Diagnostica) was diluted 1 : 500 in PBS-2% Tween 20 and incubated for 1 h at room temperature. After three 10-min washes, goat anti-mouse secondary antibody (Dako, Ely, UK) was diluted 1 : 10 000 in PBS-2% Tween 20 and applied for 1 h. After three 10-min washes the SAB complex diluted 1 : 10 000 was applied to the blot for 45 min at room temperature. The blots were washed five times for 10 min with PBS-0.05% Tween 20. Chemiluminescent detection was performed with Pierce SuperSignal West Pico Substrate (Pierce) and exposure onto X-ray film (Super RX Fugifilm).

Soluble fibrin immunoblots: Soluble fibrin was detected in 3 μL culture supernatants as described above

for D-dimers, but using a mouse monoclonal antibody against human fibrin (ADI 350, 1.0 mg/mL, American Diagnostica) diluted 1 : 1000. The fibrin antibody did not recognize fibrinogen. The specificity of the antibody was confirmed by comparing the immunostaining of fibrinogen (1 mg/mL) and fibrin (1 mg/mL fibrinogen clotted with 0.1 U thrombin in the presence of 20 mM CaCl_2) coated overnight onto microtitre plates.

Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) in supernatants and cell lysates was measured using the TOX-7 LDH assay (Sigma). Cell culture supernatants and cell lysates were diluted 1 in 10 by adding 5 μL of sample to 45 μL of PBS. To each well, 25 μL of the LDH assay mixture was added. The plate was incubated for 5 min at room temperature, and protected from light, and 8.33 μL of 1 M HCl was added to stop the reaction. The absorbance at 490 nm was measured using a microplate reader (Dynex MRX microplate reader, West Sussex, UK).

Tissue factor activity

TF activity assay was conducted on cells in a 96-well plate and the method was adapted from Lwaleed et al. [23]. 16HBE 140- cells were seeded at 25 000 cells per well in a 96-well tissue culture plate and grown to 90% confluence for 1–2 days. At confluence, the cells were washed in PBS and serum-starved overnight in MEM-ITS. The cells were washed in PBS and 100 μL of fresh MEM-ITS medium was added. The cells were wounded (one horizontal and one vertical scrape) with a 2 μL pipette tip to produce a 0.5 mm wound, and incubated for 20 min or 2 h. After the incubation period the media were removed and 60 μL 0.05 M Tris-NaCl pH 7.8 (or 60 μL of standards added to wells that contained no cells), 20 μL of 0.025 M CaCl_2 and 15 μL of 0.75 IU/mL Prothromplex TIM-4, a concentrate of human coagulation factors II, VII, IX and X (Baxter Healthcare, Thetford, Norfolk, UK), were added to the cell culture plate, which was then incubated for 15 min at 37 °C. The further generation of active coagulation factors by the extrinsic coagulation pathway was prevented by adding 100 μL 0.05 M Tris-NaCl pH 7.8 containing 7 mM EDTA. The activity of FXa was measured by the addition of 40 μL 1 mM S-2222 (Bz-Ile-Glu-Gly-Arg-pNA · HCl) chromogenic substrate. The plate was read at 405 nm (Dynex MRX microplate reader) for 1 h at 37 °C and the initial rate was calculated as the rate of change of optical density.

Neutralization of coagulation factor activity and wound closure

Mouse anti-human TF (American Diagnostica), sheep anti-human FXIIIa (Affinity Biologicals), rabbit anti-

human fibrinogen (Calbiochem, Nottingham, UK) and corresponding, non-immune mouse IgG (Sigma), sheep IgG (Sigma) and rabbit IgG (Sigma) controls were added to wells of a 24-well plate to give final concentrations of 1, 5 and 10 $\mu\text{g}/\text{mL}$ for 30 min at 37 °C. A single linear wound with a width of $\sim 500 \mu\text{m}$ was produced by scraping the monolayers with a 2 μL pipette tip across the diameter of the well. Images were obtained using a Leica microscope (Wetzlar, Germany), equipped with a Ricoh 35mm SLR camera (Ricoh Co. Ltd, Tokyo, Japan) at the initial time of wounding and 13 h post-wound for 16HBE 140- cells and 20 h for NHBE. Ten measurements were made across the wound width of each of the duplicate wells and averaged.

Reverse transcription polymerase chain reaction for tissue factor and factor VII mRNA expression

Total RNA was extracted from 16HBE 140- cells grown to confluence in six-well plates and quiesced for 18 h in 1 mL serum-free ITS-supplemented medium, as described above. The medium was refreshed prior to wounding, and confluent and wounded (eight scrapes) cell layers were incubated for up to 12 h. At each time-point investigated, total RNA was extracted using the Promega RNagents[®] (Southampton, UK) total RNA isolation system. RT-PCR for TF and the 18S RNA housekeeping gene was carried out in two steps. Reverse transcription and synthesis of cDNA using random hexamer primers (Invitrogen, Paisley, UK) was followed by PCR using the primers indicated in Table 1 and Qiagen Mastermix[®] (Crawley, UK) under conditions in which amplification of each product was linear. For TF this was 10 min at 72 °C to linearize the cDNA, and then 30 cycles of amplification consisting of denaturation at 94 °C for 20 s, annealing at 57 °C for 20 s and extension for 40 s at 72 °C. This was followed by a final extension step of 2 min at 72 °C. For 18S RNA this was 10 min at 72 °C to linearize the cDNA, and then 17 cycles of amplification consisting of denaturation at 94 °C for 20 s, annealing at 57 °C for 20 s and extension for 40 s at 72 °C. This was followed by a final extension step of 2 min at 72 °C.

RT-PCR for FVII and the β -actin house-keeping gene was carried out using the Invitrogen Superscript[™] One-Step RT-PCR system and the primers indicated in Table 1. RT-PCR reactions for FVII were subjected to 50 °C for 30 min to synthesize the cDNA and 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 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. RT-PCR reactions for β -actin were subjected to 50 °C for 30 min to synthesize the cDNA and then pre-denaturation for 2 min at 94 °C, followed by 30 cycles of amplification 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.

Table 1. PCR primers for tissue factor (TF), factor VII (FVII) and the house keeping genes 18S RNA and β -actin

Gene	Direction	Sequence	Product size (bp)	T_m (°C)
18S RNA	Forward	catgcatgtctaagtacgcacggcc	200	66.3
	Reverse	gggctgaccgggttggttt		61.4
β -actin	Forward	gcgggaaatcgtgcgtgacatt	232	57.0
	Reverse	gatggagttgaagtagtttcgtg		56.0
TF	Forward	caacagacacagagtgtgacctcaccgacg	502	66.0
	Reverse	tctctgaattccctttctctggccc		63.0
FVII	Forward	agctcatgggtgctcaacgtg	404	53.5
	Reverse	atatgggatttggtgccagg		53.6

RT-PCR products were analysed on 2% (w/v) agarose gels with a 100 bp DNA ladder (Invitrogen) and negative (no cDNA) controls were also loaded. Bands were visualized by ethidium bromide fluorescence under UV light and densitometry using SynGene software to semi-quantify the relative abundance of mRNA bands encoding each gene sequence compared with the internal control 18S RNA or β -actin.

Growth factor assays

Hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), TGF- β 1 and epidermal growth factor (EGF) were measured in culture supernatants from undamaged and maximally wounded (eight scrapes, as described above) 16HBE 14o-monolayers using Quantikine ELISA kits (R&D Systems, Abingdon, UK). prostaglandin E2 (PGE2) was measured using the Parameter High Sensitivity immunoassay (R&D Systems).

Statistical analysis

Statistical analysis was performed using GraphPad INSTAT V2.05a to carry out a one-way ANOVA with a *post hoc* Bonferroni's test.

Results

Tissue factor

TF was constitutively expressed by resting confluent monolayers, and appeared to be concentrated at the periphery of the cells (Fig. 1), in keeping with its trans-membrane distribution [19]. TF activity was strongly detected on the surface of confluent monolayers, and the concentration measured by ELISA in cell lysates was 152.1 ± 20.6 ng/ 10^6 cells. Neither TF activity nor the concentration changed significantly 20 min or 2 h after wounding (data not shown). However, 1 h after wounding, there was an increase in the intensity of TF staining in cells at the edge of the wound (Fig. 1c) and these cells adopted a polarized morphology, extending pseudopodia

towards the centre of the wound (Fig. 1d). FXIII had a cytosolic distribution in the confluent monolayers (Fig. 1b) and again appeared to be more abundant at the wound edge following wounding (not shown).

Initial experiments demonstrated that wound closure was due to cell migration and not proliferation, as reported previously [24]. The rate of repair of 16HBE cell layers was not significantly different in the absence and presence of mitomycin C (1 μ g/mL) to inhibit proliferation. The gap remaining 9 h after wounding was $20.2 \pm 4.7\%$ and $15.4 \pm 2.2\%$ of the width of the original wound ($n = 8$) in the absence and presence of mitomycin C, respectively.

A neutralizing antibody was used to investigate the role of TF in the repair of wounded 16HBE 14o- monolayers (Fig. 2a). In the absence of the antibody, the wound had almost completely closed 13 h after wounding. The gap remaining at this time-point was $1.2 \pm 0.6\%$ of the width of the original wound. TF antibody at 1 μ g/mL significantly inhibited the repair response, and the wound remained open at $84.6 \pm 2.4\%$ of the original wound width 13 h after wounding. Higher concentrations of the antibody had no further inhibitory effect, and non-immune mouse IgG had no inhibitory effect on wound repair.

TF not only activates the extrinsic coagulation cascade but also generates proteases that activate protease-activated receptors (PARs) present on airway epithelial cells [25, 26]. A direct role for cross-linked fibrin formation in the wound repair process was therefore investigated using antibodies to fibrinogen and FXIIIa. These antibodies also significantly inhibited wound repair. At 13 h after wounding, the wound remained open at $72.7 \pm 7.8\%$ and $78.5 \pm 6.1\%$ of the original gap width in the presence of 1 μ g/mL anti-fibrinogen and anti-FXIIIa, respectively. Higher concentrations of the antibodies had no further inhibitory effect and control Igs were without an inhibitory effect on wound repair.

In primary NHBE cell cultures, the wound remained partially open ($31.61 \pm 0.96\%$ of the original wound width) in control wells even after 20-h culture (Fig. 2b). However, neutralizing antibodies to the coagulation

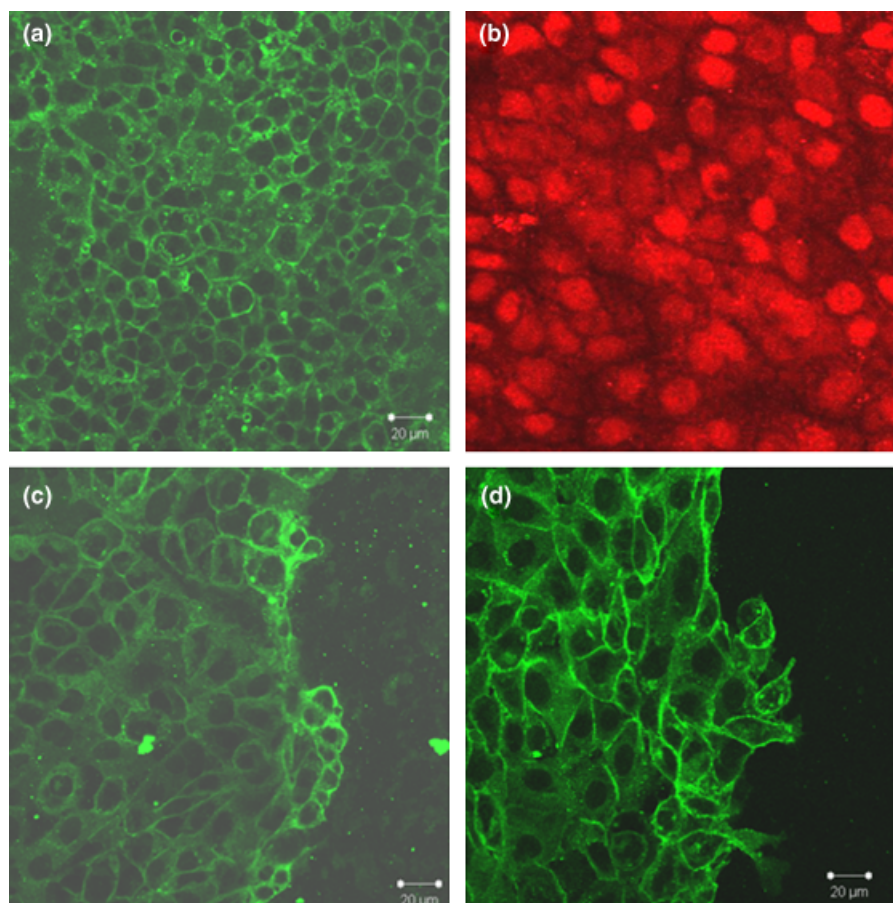


Fig. 1. Immunostaining of 16HBE cell monolayers for TF (a, c, d) and FXIII (b). Confluent cell monolayers were stained for TF (a) and FXIII (b), or were stained for TF 1-h post-wounding (c, d). Controls lacking the primary or secondary antibody were negative in all cases. TF, tissue factor; FXIII, factor XIII.

factors TF, fibrinogen and FXIIIa significantly inhibited wound closure in these cultures, while control Igs had no effect (Fig. 2b).

The coagulation cascade depends on the formation of an initiation complex between TF and FVII. We therefore investigated the expression of TF and FVII at the mRNA level over the time course of wound closure. Transcripts for both TF and FVII were detected in confluent 16HBE 140- cell monolayers (Fig. 3) and their expression increased significantly post-wounding (Fig. 3). A significant increase in TF mRNA was not detected until 10 h after wounding (Fig. 3b) and a significant, relatively rapid, increase in FVII mRNA expression was detected 4 h post-wounding (Fig. 3d).

However, in view of the very rapid formation of fibrin in response to bronchial epithelial wounding reported *in vivo* [1], changes in the abundance of FVII at the protein level were investigated over a much shorter time course, 20 min and 2 h, following wounding. Preliminary experiments using Western blotting had detected FVII as both a high-molecular-weight complex with TF, as tryptic fragments of 20 and 35 kDa, as reported previously [27], and

lower molecular weight fragments. A cocktail of protease inhibitors added after harvesting cell culture supernatants did not prevent the proteolytic changes, and could not be added during wounding experiments because all the coagulation factors, except FXIII, are serine proteases. Therefore FVII, in all its forms, was detected by staining immunoblots using a polyclonal antibody (Fig. 4a) and this approach was used for the detection of FX (Fig. 4b), fibrinogen (Fig. 4c) and FXIIIa (Fig. 4d).

FVII (Fig. 4a) was undetectable in supernatants of control wells, despite the detectable presence of mRNA transcripts (Fig. 3d) indicating that confluent epithelial cells do not constitutively release this coagulation factor. However, when the cell layers were wounded, FVII was readily detectable in the cell supernatant 20 min and 2 h after scrape damage. The highest concentration of FVII (0.37 ± 0.1 ng/mL) was already detectable 20 min after the most extensive level of wounding.

Constitutive expression of FX (Fig. 4b) was detectable in 2-h cultures (0.63 ± 0.18 ng/mL). Although FX was undetectable in supernatants of confluent monolayers cultured for 20 min, wounding induced detectable levels

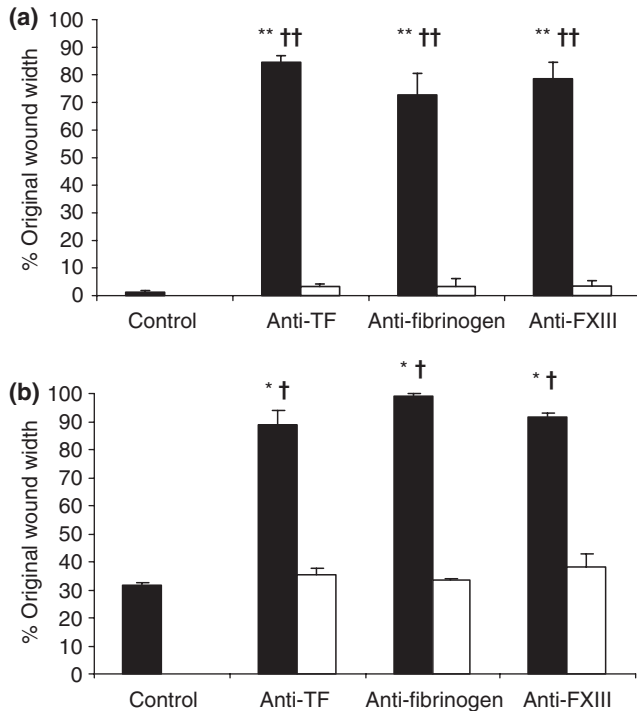


Fig. 2. Effect of coagulation factor neutralizing antibodies on wound repair in 16HBE 140- and primary HBE monolayers. Black bars indicate the effects of no-antibody control, anti-TF (1 µg/mL), anti-fibrinogen (1 µg/mL) and anti-FXIII (1 µg/mL) on the % original wound width of (a) 16HBE 140- cells at 13 h and (b) primary HBE at 20 h. White bars represent the effects of corresponding non-immune mouse, rabbit and sheep Igs (1 µg/mL). Data represent mean ± SEM ($n = 3$ independent experiments). * $P < 0.001$, ** $P < 0.0001$ compared with no-antibody control and † $P < 0.05$, †† $P < 0.0001$ compared with non-immune Igs. TF, tissue factor; FXIII, factor XIII.

of FX that increased with the extent of wounding, and the maximum levels detected were not different at 20 min or 2 h.

Fibrinogen (Fig. 4c) was not detected in culture supernatants of undamaged monolayers, but wounding rapidly induced a detectable level of fibrinogen, with fibrinogen levels increasing in relation to the extent of damage. Fibrinogen levels in supernatants harvested 20 min and 2 h post-wounding were not significantly different. However, when wound repair was inhibited by anti-TF and anti-FXIII as described above (Fig. 2), there was a significant accumulation of fibrinogen in the medium, indicating that fibrinogen is not only released in response to wounding but also consumed in the repair process (Fig. 5).

The cellular transglutaminase that cross-links soluble fibrin to form an insoluble clot, FXIIIa, was undetectable in undamaged cell culture supernatants (Fig. 4d). However, this coagulation factor rapidly became detectable following damage to the cell layer. The response was greatest after 20 min, to a maximum of 125.6 ± 47.0 ng/mL with eight scrapes.

D-dimer formation is evidence of both fibrinogenesis and fibrinolysis. D-dimers were detected at baseline and

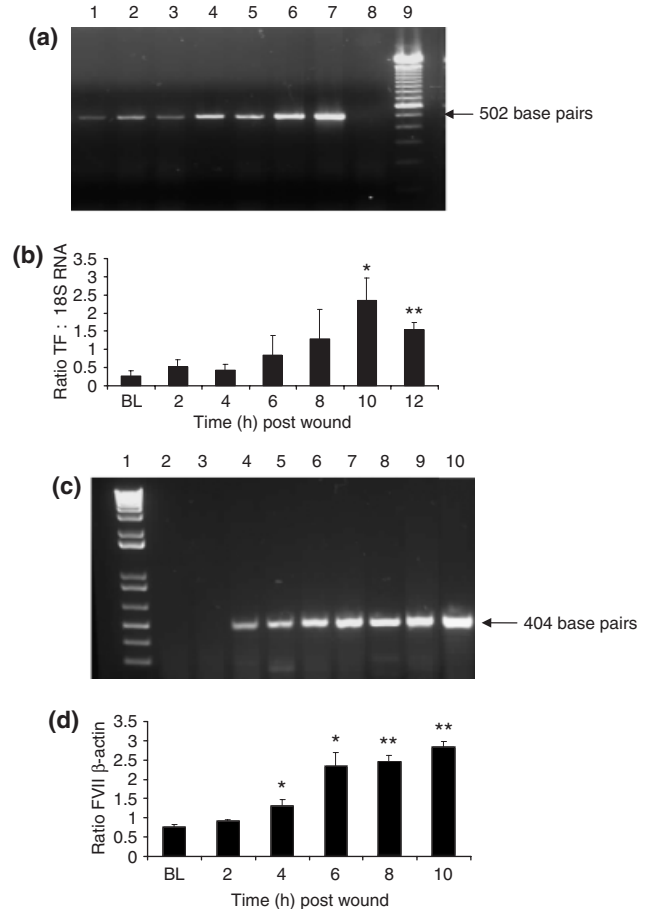


Fig. 3. RT-PCR analysis of TF (a, b) and FVII (c, d) mRNA transcripts in confluent and wounded 16HBE monolayers. (a) (1) Confluent layers and (2) 2 h, (3) 4 h, (4) 6 h, (5) 8 h, (6) 10 h, (7) 12 h post-wounding, (8) negative control (no reverse transcription), (9) 100 bp ladder. (b) Ratio of TF to the 18S RNA internal control, mean ± SEM ($n = 4$). (c) (1) 100 bp ladder, (2) negative control (no RNA), (3) negative control (no reverse transcription), (4) confluent layers and (5) 2 h, (6) 4 h, (7) 6 h, (8) 8 h, (9) 10 h post-wound, (10) liver RNA (positive control). (d) Ratio of FVII to the β-actin internal control, mean ± SEM ($n = 3$). BL, confluent monolayers. * $P < 0.05$ and ** $P < 0.0005$ compared with BL. TF, tissue factor; FVII, factor VII.

the levels increased significantly with increasing extent of damage (Fig. 4e). D-dimer formation was maximal at 20 min (2.4 ± 0.19 ng/mL) and was not further increased at 2 h after the greatest degree of wounding to the monolayers.

Soluble fibrin monomers, that form when fibrinopeptides are cleaved but before the protein is cross-linked, were detected in supernatants of confluent monolayers in the absence of plasma, and significantly higher levels were detected 20 min following mechanical damage that increased with the extent of wounding and were not different from those detected at 2 h (Fig. 4f).

The time course indicates that the rapid release of pre-formed proteins initiates the repair response that is

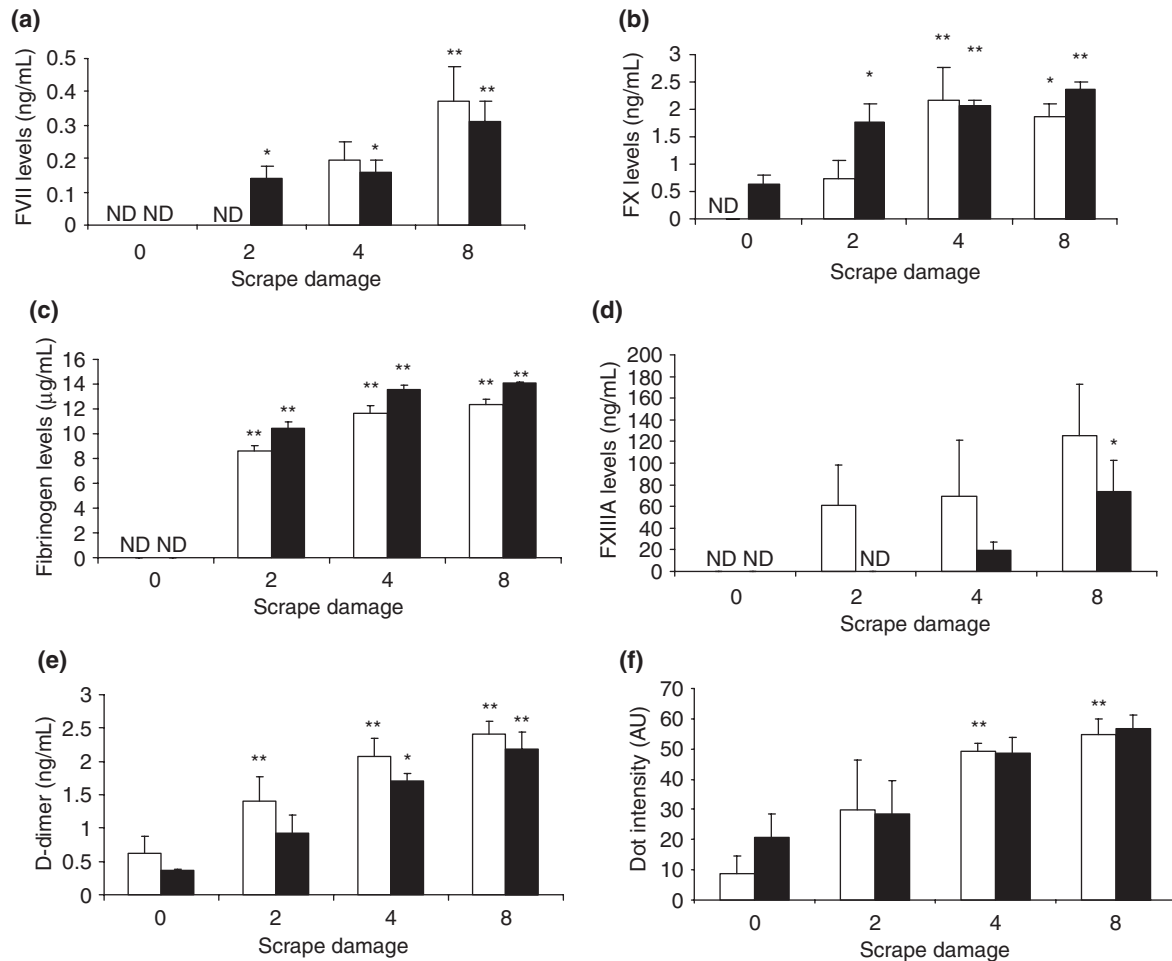


Fig. 4. Immunoblot analysis of FVII (a), FX (b), fibrinogen (c), FXIIIa (d), D-dimer (e) and soluble fibrin (f) levels in 16HBE cell supernatants 20 min (white bars) and 2 h (black bars) after scrape damage. Results are given as mean $\pm$ SEM for $n = 3-4$ independent experiments. Significance was assessed by comparison with the control values * $P \leq 0.05$, ** $P \leq 0.01$ (ND, not detected). TF, tissue factor; FVII, factor VII; FX, factor X; FXIIIa, factor XIII subunit A.

sustained by increase in the expression of coagulation factors over the longer time course to wound closure. LDH activity in supernatants from undamaged 16HBE 140- cell layers was 1% of the total measured in cell lysates, and there was no significant increase 2 h after any degree of wounding. Thus, mechanisms more specific than release from damaged or dead cells were responsible for the response. In addition, the potential contribution from other known mediators of epithelial repair, PGE₂, HGF, EGF, KGF and TGF- β 1, was investigated in our model, but all were detected at very low levels, at the limit of detection in the assays, and levels too low to participate in the repair process (Table 2). Only TGF- β 1 increased significantly following wounding, although this was an increase in latent TGF- β 1 and no increase in active TGF- β 1 was detected.

In conclusion, the data clearly indicate a role for coagulation factors expressed by bronchial epithelial

cells, and released in response to wounding, in the bronchial epithelial repair process.

Discussion

This study has demonstrated for the first time that bronchial epithelial cells in culture express FVII, FX, fibrinogen and FXIIIa, and are able to synthesize fibrin in the absence of plasma. We have shown the release of FVII, FX, fibrinogen and FXIIIa following mechanical wounding of bronchial epithelial cell monolayers. Two lines of evidence point to the capacity of these cells to generate a fibrin matrix in the absence of plasma. Firstly, detection of soluble fibrin monomers in culture supernatants and, secondly, D-dimer formation, indicative of sequential fibrinogenesis and fibrinolysis. Our results also indicate that locally produced coagulation cascade proteins and the formation of insoluble cross-linked fibrin

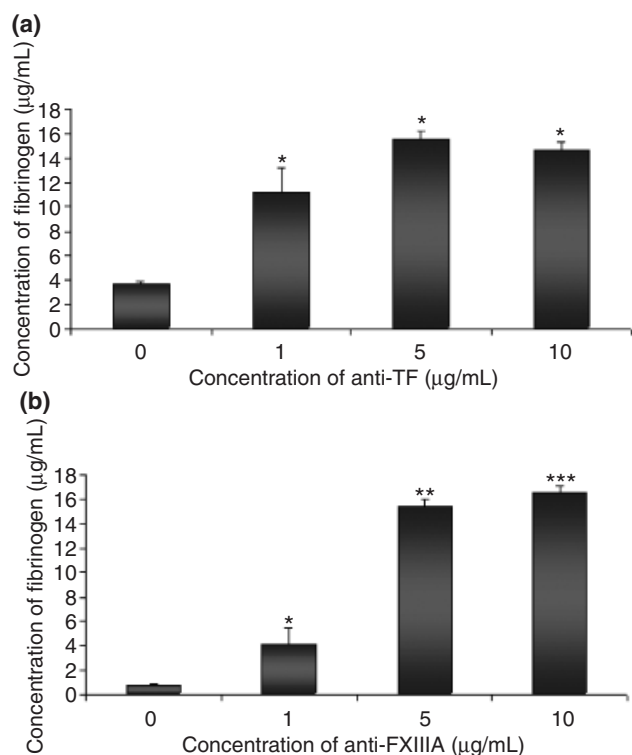


Fig. 5. Concentration of fibrinogen in cell culture supernatants from 16HBE 14o- cells 13 h post-wounding, following inhibition of wound repair by anti-TF (a) and anti-FXIIIa (b), as described in Fig. 2. Data represent mean $\pm$ SEM ($n = 3$). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$ compared with no antibody control. TF, tissue factor; FXIIIa, factor XIII subunit A.

matrices are essential for normal epithelial repair and is independent of plasma proteins.

TF is the exclusive activator of the extrinsic coagulation pathway and is predominantly expressed on the cell surface where it binds FVII, acting as a co-factor to enhance its proteolytic activity [19]. Immunohistochemical analysis of normal human tissues previously demonstrated TF expression in the bronchial and alveolar epithelium [18], and TF activity on NHBE cells and alveolar A549 cells in culture has been reported [28]. TF exists in microdomains on the cell surface with a mixed population of active and encrypted protein, encrypted meaning that while the TF antigen is detectable on the surface of unactivated cells, its full pro-coagulant activity is not [29]. FVIIa is able to bind to both encrypted and de-encrypted TF, but when the TF is encrypted the TF/FVIIa complex activates cell signalling, but does not activate FVII/FX and subsequent coagulation [30, 31]. De-encryption, i.e. without a change in TF mRNA or protein, occurs in response to a number of stimuli, including cell damage, proteases, phospholipases, apoptosis, complement and calcium ionophores [29, 31]. Despite the evidence for a rapid increase in coagulation and fibrin formation following damage, we were unable to detect a change in TF activity, measured as the generation

Table 2. Concentration of growth factors in the supernatant of confluent and wounded cell layers

Growth factor	Confluent	Wounded	
		2 h	12 h
EGF (pg/mL) ($n = 5$)	0.123 $\pm$ 0.01	0.256 $\pm$ 0.01	0.063 $\pm$ 0.01
KGF (pg/mL) ($n = 5$)	13.86 $\pm$ 2.20	10.11 $\pm$ 4.66	12.86 $\pm$ 2.97
Total TGF- β 1 (pg/mL) $n = 5$	16.75 $\pm$ 1.68	19.70 $\pm$ 1.78	29.98 $\pm$ 2.78*
HGF (pg/mL) ($n = 3$)	ND	ND	–
PGE2 (pg/mL) ($n = 3$)	9.53 $\pm$ 0.34	9.79 $\pm$ 0.52	–

* $P < 0.05$ compared to confluent layers.

ND, non-detected; –, not measured; EGF, epidermal growth factor; KGF, keratinocyte growth factor; HGF, hepatocyte growth factor; PGE2, prostaglandin E2.

of active Xa, with wounding. It is possible, however, that only TF in cells at the wound edge, representing a small proportion of the total number of cells, became de-encrypted, rapidly switching function to promote coagulation. The increase in TF immunostaining at the wound edge 1 h post-wounding may represent de-encryption, as TF dimers encrypted in lipid rafts dissociate after cell stimulation and are released into the liquid disordered phase of the membrane [29]. TF expression is rapidly induced (within 1 h) in endothelial cells [25], but no changes in TF protein or mRNA levels were detected over 2 h in wounded bronchial epithelial cell layers. Instead, TF mRNA expression was significantly increased over a longer time course, at 10 and 12 h post-wounding.

TF is both constitutively active and functionally important in wound repair, because neutralization of this activity resulted in significant inhibition of the repair response. TF not only initiates the extrinsic coagulation cascade leading to fibrin formation, but generates FVIIa, FXa and thrombin, activators of PARs that are present on the bronchial epithelium [25, 26]. However, a direct association of the repair response with the coagulation cascade and fibrin formation was demonstrated using blocking antibodies to fibrinogen and FXIIIa to inhibit the repair response. In addition, fibrinogen released in response to wounding accumulated in culture supernatants when repair was inhibited. The data confirmed that a cross-linked fibrin matrix is essential for normal epithelial repair under the conditions used in our experiments. Hence, in the absence of plasma, it appeared that the bronchial epithelial cells were the source of all the coagulation factors involved in the cascade leading to fibrin formation. The presence of FVII, FX, fibrinogen and FXIIIa in cell culture supernatants following wounding supported this notion.

Expressions of FVII and FX were once believed to be hepatocyte specific. However, FVII has also been demonstrated in macrophages, endothelial cells, smooth muscle cells, fibroblasts and keratinocytes [32]. The release of

FVII and the increase in FVII expression with wounding in our experiments are indicative of a repair response. Preliminary experiments (data not shown) have indicated that FVII associates with TF on the cell surface to form an initiation complex. The presence of active FVII on NHBE cells was previously indicated in a study demonstrating TF-mediated coagulation in FVII-depleted plasma [28]. Similarly, previous studies using immunohistochemical staining localized active FX to the nasal and bronchial epithelium as well as in macrophages [33]. In addition, FXa/FXa-like activity has been demonstrated on the surface of alveolar A549 cells [34], and it was recently reported that the alveolar epithelium and small airway epithelial cells can initiate the extrinsic coagulation cascade through expression of TF activity [35].

Vitamin K-dependent carboxylase is essential for introducing post-translational modifications that are required for Ca^{2+} binding and functional activity of coagulation factors. The liver was once thought to be the sole source of vitamin K-dependent carboxylase, but it has been detected in a number of tissues including the lung. The presence of this enzyme in bronchial epithelial cells would support the notion that coagulation factors can be synthesized and released in active form by this cell type. Evidence that this is the case is provided by a study showing release of activated protein C, a carboxylation-dependent regulatory protein, from bronchial epithelial cells [36].

Fibrinogen was previously shown to be expressed by the alveolar epithelium [37]. In our study, constitutive release of fibrinogen into bronchial epithelial cell culture supernatants was not detected, although its release was rapidly induced following mechanical damage, suggesting that bronchial epithelial cells contain pre-formed protein. These observations parallel those recently described for alveolar epithelial cells, in which expression of fibrinogen was dependent on an inflammatory stimulus and which could accumulate protein prior to release [38]. The accumulation of fibrinogen in supernatants of wounded primary and 16HBE 14o- cell cultures when coagulation factor activity and repair were inhibited indicated that it is normally consumed in the repair process. The release of fibrinogen, in conjunction with coagulation factors, suggests that it may play a role in fibrin formation, although fibrinogen *per se* may also constitute part of the extracellular matrix (ECM) [22].

FXIII is a member of the transglutaminase family of enzymes and FXIII-A is the subunit responsible for the catalytic function of the factor [39]. Extracellular FXIII is activated by thrombin to FXIIIa, generating transglutaminase activity that cross-links fibrin monomers and is highly influential in determining the stability of a newly formed provisional matrix and promoting tissue repair [40]. FXIIIa also forms covalent cross-links between fibrin, collagen and fibronectin, providing a mechanism

to anchor the clot to the ECM and therefore keep it at the site at which it is required [41]. Additionally, FXIII has the potential to facilitate repair by acting as an adhesion molecule for a number of cell types, and these interactions are important in anchoring lung epithelial cells to ECM proteins [42]. More recently, FXIII was demonstrated to be a ligand for the integrins $\alpha_9\beta_1$ and $\alpha_4\beta_1$ [43], of which the integrin $\alpha_9\beta_1$ is highly expressed on basal bronchial epithelial cells [44].

FXIIIa had a cytosolic distribution in resting cells, but damage to confluent bronchial epithelial cell layers led to the release of FXIIIa into the culture supernatants. Similarly, in other studies, tissue transglutaminase was released when fibroblast layers were mechanically damaged, but became bound to the ECM [45]. The lower levels of FXIII detected 2 h compared with 20 min post-wounding in our experiments may therefore indicate binding of FXIII to matrix proteins and/or degradation, as shown for fibroblast cultures over 2 h [46]. The recent discovery of PAR-1-stimulated synthesis and release of FXIII suggests that more specific mechanisms may also exist [47]. We have now established that PAR-1 and PAR-2 peptide agonists are stimuli for fibrinogen and FXIIIa release, D-dimer formation and an increased rate of bronchial epithelial repair [48] (and manuscript in preparation).

Provisional matrices contain a mixture of fibrinogen and insoluble fibrin, which is removed by plasmin-mediated proteolysis. The detection of D-dimers in the cell culture supernatants in response to damage provides evidence for plasmin-mediated proteolysis of insoluble fibrin. Because D-dimer is formed during thrombus formation when FXIIIa cross-links the terminal D-domains of fibrin, the presence of D-dimers is indicative of both fibrinogenesis and fibrinolysis, and is used clinically as an indicator of *in vivo* fibrin formation. Thus, it appears that fibrin formation on bronchial epithelial cells is a dynamic process, with production and dissolution occurring simultaneously. In parallel with our observations, it was interesting to note that inhibition of fibrinolytic activity may enhance the repair of alveolar epithelial cells grown on fibrin matrices [49].

Previous studies have indicated a role for PGE2 (10 $\mu\text{g/mL}$) [50] and growth factors added exogenously, including EGF (10 ng/mL) [51], HGF (20 ng/mL) [52], TGF- β_1 (250 pg/mL) [24] and KGF (10 ng/mL) [53] in the repair of airway epithelia. However, the concentrations of these factors produced endogenously in our model were too low to contribute to the observed repair response. Serum-free conditions limit prostaglandin production [54, 55] and may limit the production of other growth factors. Although we have not ruled out a role for ATP [56], NO [57] or other growth factors in the repair response, our reductive model has revealed the previously unrecognized functional importance of locally produced coagulation

factors in normal bronchial epithelial repair and the potential of the bronchial epithelium to produce a provisional fibrin matrix independently of plasma proteins.

While plasma exudation follows epithelial injury in a number of settings including the response to mechanical damage [58], our observations are likely to be important *in vivo* when plasma exudation does not follow damage, or is limited pharmacologically by the anti-inflammatory action of corticosteroids and the direct effect of β_2 -agonists on the endothelial cells of post-capillary venules [59]. For example, plasma exudation is a feature of the inflamed asthmatic airway that is inhibited by inhaled corticosteroid therapy, without effects on local protein synthesis [12]. A study of the effect of prednisone in patients with severe persistent asthma indicated no significant change in the levels of fibrinogen in the airways, suggesting a local cellular source of fibrinogen [60]. Other studies have shown that allergen challenge of the airways induced leakage of plasma proteins 4 h after challenge only in subjects with allergic asthma and not in healthy controls or non-asthmatic subjects with allergy [61]. A significant increase in plasma proteins in the airway was not observed 5 min after challenge in the asthma group, suggesting that rapid fibrin-dependent repair may initially depend on locally produced coagulation factors to initiate the process. Our results indicate that autonomous repair of the normal bronchial epithelium proceeds via fibrinogenesis and fibrinolysis acting in concert to limit fibrin formation. TF-bearing cells are believed to be involved only in the initiation of clot formation, while the growth of the clot is dependent on the propagation of coagulation to bulk plasma [62]. Thus, while plasma exudation is normally considered to be a defensive repair response [1, 3, 58, 59], plasma exudation may become a pathophysiological event supporting excessive fibrin formation in conditions such as ARDS [15], acute lung injury [16] and status asthmaticus [17]. The pro-coagulant activity of culture supernatants from 16HBE cells exposed to particulate matter was recently indicated [63] and in preliminary experiments we have found increased expression of FVII and FXIII following exposure to endotoxin. Thus, in addition to a mechanical stimulus, exposure to a number of airborne factors may stimulate the coagulation pathway, and contribute to tissue remodelling in the airway. A recent report indicated that FXa is involved in airway remodelling in asthma [64]. We now propose a role for the extrinsic coagulation cascade in the repair response of normal primary bronchial epithelial cells that supports the notion that a defect in this pathway may underlie defective epithelial repair in asthma [65].

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