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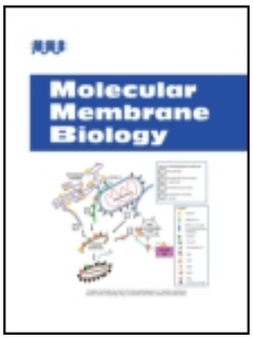

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Peptidase allergen Der p 1 initiates apoptosis of epithelial cells independently of tight junction proteolysis

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Summary

Loss of epithelial cell polarity, which can arise following disruption of tight junctions (TJs), is a precursor to the carefully orchestrated removal of moribund cells from epithelia in apoptosis. Ordinarily, this cycle of events has minimally disruptive effects on the function of the epithelial barrier, but some agents have been identified that induce apoptosis and promote epithelial leakiness. The allergen Der p 1 is a cysteine peptidase that cleaves TJ adhesion proteins and induces apoptosis in epithelial cells. This suggests the possibility that, at least for some inducers of apoptosis, these events might be causally linked. We report here that Der p 1 induces epithelial apoptosis before outright cell detachment and that apoptosis occurs within the same time span as increased paracellular permeability in polarized epithelial monolayers. Whilst TJ-deficient BEAS-2B cells were resistant to Der p 1-induced apoptosis, the cell line 1HAEo-, which was also TJ deficient, was sensitive to Der p 1, providing evidence against TJ proteolysis as a cause of apoptosis. To provide direct evidence, we propagated cells that normally express TJs in low calcium medium that prevented intercellular junction assembly. These cells retained full susceptibility to Der p 1, indicating that Der p 1-induced apoptosis is independent from TJ proteolysis.

Keywords: Adhesion molecules; lung; mucosa; apoptosis; allergy.

Introduction

Adhesive contacts between cells and with matrix proteins prevent apoptosis [1–5]. In apoptosis these contacts are disrupted, cells become rounded and the cytoskeleton is rearranged into a ring before blebbing [6]. Apoptosis can arise from the progressive development of these events or by an outright loss of the spatial cues normally provided by cell–cell and cell–matrix adhesion in a specialized type of apoptosis known as anoikis [6–8].

The mechanisms linking apoptosis and disrupted cell adhesion are not fully established, but the cytoskeleton is implicated as a possible transducer and target [9–11]. Activation of caspase-8, a crucial initiator of the death receptor pathway, is accompanied by degradation of the cytoskeleton [10] and actin is a likely substrate for caspases [12]. Additionally, evidence indicates that the cytoskeleton can function as a direct initiator of cell death [13].

Tight junctions (TJs), the most apical of interepithelial adhesions responsible for regulating paracellular permeability, are affected by disruption of the cytoskeleton [14] demonstrating that intercellular adhesion is sensitive to intracellular events. In turn, the cytoskeleton responds to external perturbation of intercellular contacts by the formation of stress fibres, and the removal or degradation of cytoplasmic components of junctions. This suggests that intercellular adhesions also transduce responses from the cell surface. In this regard, several cytoplasmic signalling molecules potentially confer bidirectional signal transduction capabilities on TJs. These include two forms of heterotrimeric G proteins [15,16], PKCζ [17], PKCλ [18], the atypical PKC isotype-specific interacting protein, ASIP [19], tyrosine kinases (c-Src, c-Yes), and phospholipase C [20]. The TJ plaque proteins ZO-1, -2 and -3 are membrane-associated guanylate kinase homologues (MAGUKs), a family of proteins with SH3 and PDZ domains that tether actin filaments at cell membranes and which may couple extracellular signalling events to the cytoskeleton [20]. TJs are associated with the apical junctional actomyosin ring of epithelia and the TJ complex contains the Ras superfamily members RhoA, Rac1 and Cdc42 that control the actin cytoskeleton and regulate TJ formation [21–23]. Several of these GTPases converge on effectors such as p21-activated kinase 2 (Pak2), which is a caspase activation target for cytoskeletal changes in apoptosis [6].

These observations suggest that the disruption of TJs should be investigated as a possible means of initiating apoptosis. Of the various means of causing TJ disruption, cleavage of TJ adhesion proteins by extracellular peptidases is of interest because of its relevance to various pathophysiological settings in which the vital barrier function of epithelia is compromised. We have established that the cysteine peptidase Der p 1 and the serine peptidases Der p 3, Der p 6 and Der p 9, all of which are important environmental allergens, induce proteolysis of the TJ adhesion protein occludin, leading to increased in epithelial permeability [24–26]. Complex changes in the actin cytoskeleton accompany TJ cleavage by Der p 1 [26]. In some cells, the cortical F-actin ring becomes more prominent, whilst stress fibre formation and cell stretching occurs in areas of cell detachment [26].

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Der p 1-induced cell loss causes apoptosis, with the cells developing positive staining with annexin V and exhibiting caspase-activated DNase-dependent genomic DNA laddering. These effects of Der p 1 can be inhibited by E-64, an inhibitor of many cysteine peptidases including Der p 1 [27], but which does not inhibit the intracellular caspases involved in programmed cell death [28]. Although outlining a potentially unusual path to apoptosis, prior work has not addressed the significance of TJ proteolysis in this response. Accordingly, the present investigation used Der p 1 as a paradigm to establish if extracellular proteolysis of TJs is an initiator of apoptosis. Studies were performed using a variety of cell lines. The MDCK line was used because it is a well-characterized model of intercellular adhesion. In addition, the process was investigated in bronchial epithelial cells because the lung is a key organ target of Der p 1 in allergic disease. Studies with bronchial epithelial cells were performed in the 16HBE14o- cell line that has normal intercellular junctions [29]. For comparative purposes [30], we characterized other bronchial epithelial cell lines to investigate if those exhibiting compositional/functional deficits in their intercellular adhesions could be used to elucidate the possible contribution of TJ cleavage to apoptosis.

Results

Preliminary studies indicated that apically applied Der p 1 caused progressive loss of anchorage in MDCK and bronchial epithelial cells. Cells that appeared in free suspension were apoptotic as judged by DNA laddering. The amount of laddered DNA was proportional to the duration of Der p 1 exposure and the extent of cell loss from the monolayers. There was no evidence of overt cytotoxicity of the allergen according to LDH release.

Less aggressive treatment reduced the amount of cell detachment, but a proportion of cells nevertheless were apoptotic as judged by positive annexin V staining (designated AV^+ cells) and TUNEL labelling. Apoptotic cells remained associated with cell monolayers, developed a rounded morphology and occurred sporadically within areas of mainly non-apoptotic cells. Occasionally, apoptotic cells were found adjacent to apoptotic bodies or areas of outright cell loss. Regardless of their location, the intact apoptotic cells could not be removed by washing, indicating that although their adhesion was altered they retained a significant association with the cell monolayer and/or growth surface. This apoptotic response was explored further using conditions where $>95\%$ of cells were resistant to removal by washing.

Differential susceptibility of epithelial cell lines to allergen-induced apoptosis

Examination of several cell lines revealed a spectrum of responses to apically applied Der p 1. These ranged from MDCK cells, which were most responsive under the conditions examined, to BEAS-2B cells, which were unresponsive (Figure 1A). Figure 1B shows annexin V staining (AV^+) of these cell lines. Visual inspection of cells following exposure to Der p 1 showed that green AV^+ staining was detectable in 16HBE14o- and MDCK cell lines, with only few red PI^+ staining cells (Figure 1B). In contrast, only red PI^+ staining was observed in BEAS-2B cells (Figure 1B). This qualitative impression was confirmed by counting cells. Der p 1 neither induced necrosis as judged by propidium iodide staining alone (PI^+), nor did it increase the small number of cells that stained spontaneously with both markers (designated AV^+PI^+ cells) (Figure 1C). However, the failure of BEAS-2B cells to become either AV^+ or AV^+PI^+ after Der p 1 treatment was not due to a general resistance to apoptosis. Camptothecin, a DNA topoisomerase I inhibitor that promotes DNA cleavage and activates apoptosis, established the ability of BEAS-2B cells to become apoptotic after appropriate stimulation (Figure 1D).

Time course of AV^+ staining, TJ cleavage and increased paracellular permeability

Judged by AV^+ staining, apoptosis developed progressively in MDCK and 16HBE14o- cells before reaching an apparent plateau (Figure 2A), whereas the number of PI^+ or AV^+PI^+ cells remained constant throughout. These results also suggest that the resistance of BEAS-2B cells to Der p 1-induced apoptosis is unlikely to be due to a delayed activation of the response (Figure 2A). AV^+ staining (Figure 2A), increased paracellular permeability (Figure 2B), and a reduction in both the intensity and contiguity of the staining pattern of claudin-1 and occludin (Figure 2C) all occurred within 3 h of allergen exposure in MDCK and 16HBE14o- cells, consistent with possible linkage between these events. However, an important difference existed in the distribution of AV^+ cells and those showing signs of TJ disruption. Although the extent of allergen-induced TJ breakage varied from cell to cell, generally it could be observed throughout entire monolayers. In contrast, apoptosis occurred focally and the distribution of cells bore no obvious resemblance to the more widespread changes in TJs.

Peptidase allergen-induced apoptosis is initiated by proteolysis and is distinct from the Fas death receptor pathway

Heat inactivation abrogated the ability of Der p 1 to induce apoptosis (Figure 3A) and the epithelial permeability change (data not shown). Two other allergenic peptidases, papain (like Der p 1, a cysteine peptidase) and trypsin (a serine peptidase), also promoted the development of AV^+ staining (Figure 3B), but as with Der p 1 there was no significant change in PI^+ or AV^+/PI^+ staining cells.

To ascertain if Der p 1-induced cell death via Fas ligation or TNF/TNFR signalling, we sought transcripts encoding key components of these classical pro-apoptotic pathways (Figure 3C). Fas transcripts were found in 16HBE14o-, 1HAEo- and BEAS-2B cells in the absence and presence of allergen, whereas Fas ligand was only detectable in 16HBE14o- cells treated with PMA and in the Jurkat cell-positive control. These findings suggest that Fas ligation is unlikely to trigger the response to Der p 1 in epithelial cells. In contrast, transcripts for TNFα appeared to reflect their ability to

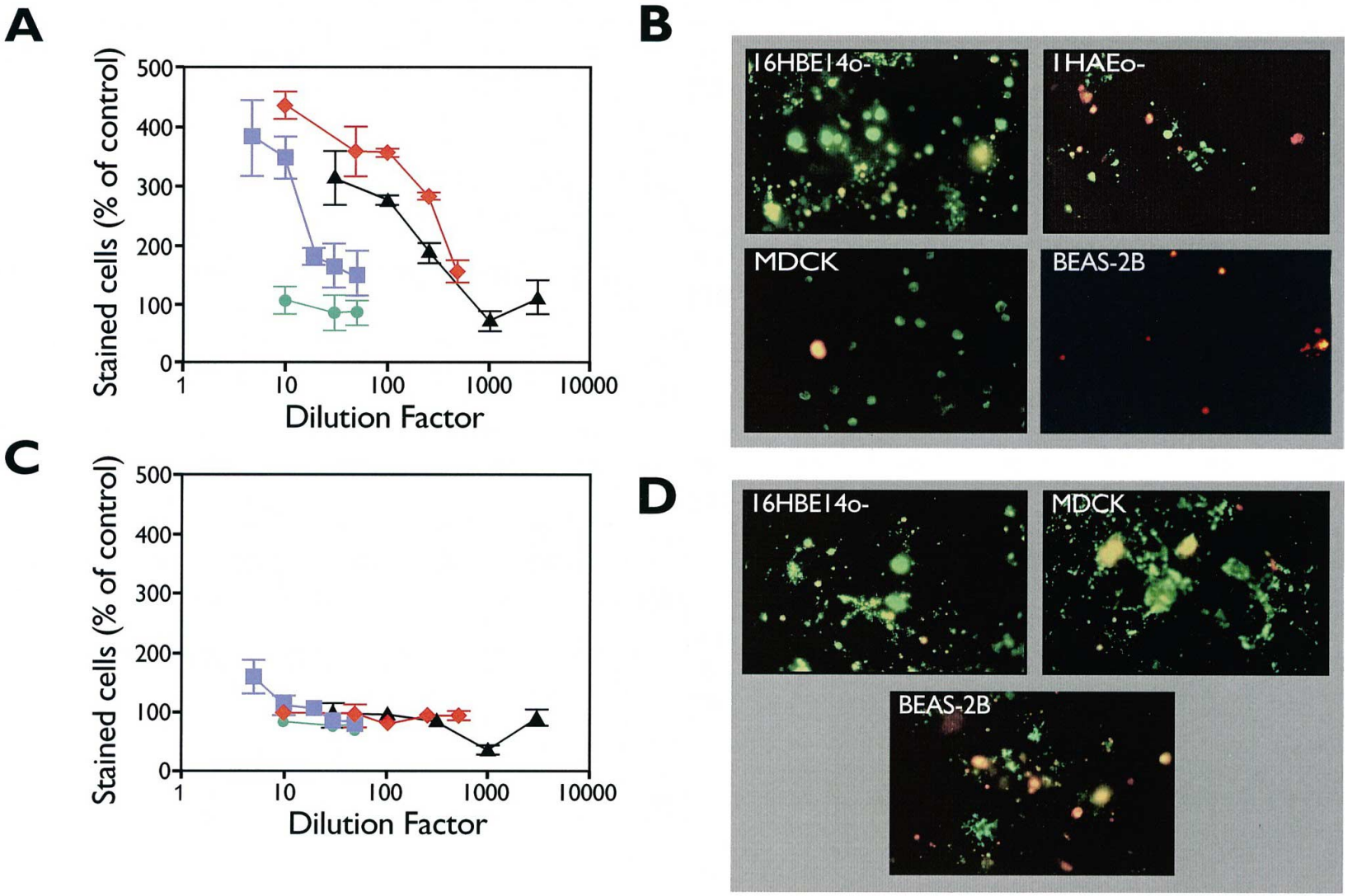

Figure 1. Concentration-dependent induction of apoptosis in epithelial cell lines by Der p 1. AV^+ staining of BEAS-2B cells (green circles), 16HBE14o- cells (blue squares), 1HAEo- cells (black triangles) and MDCK cells (red diamonds) 1 h after apical application of the indicated dilutions of Der p 1 activated by 0.5 mM GSH (A). Data are the mean $\pm$ SE mean of three experiments. Staining patterns of the four cell lines are shown in (B). Data for cells in the same cultures examined in (A) that were stained with either PI^+ or AV^+/PI^+ are shown in (C). In both (A) and (C) a dilution of 1:100 of Der p 1 corresponds to the enzymatic activity contained in 50 house dust mite faecal pellets after ageing [24]. (D) Staining responses of cells treated for 4 h with 10 μM camptothecin for comparison.

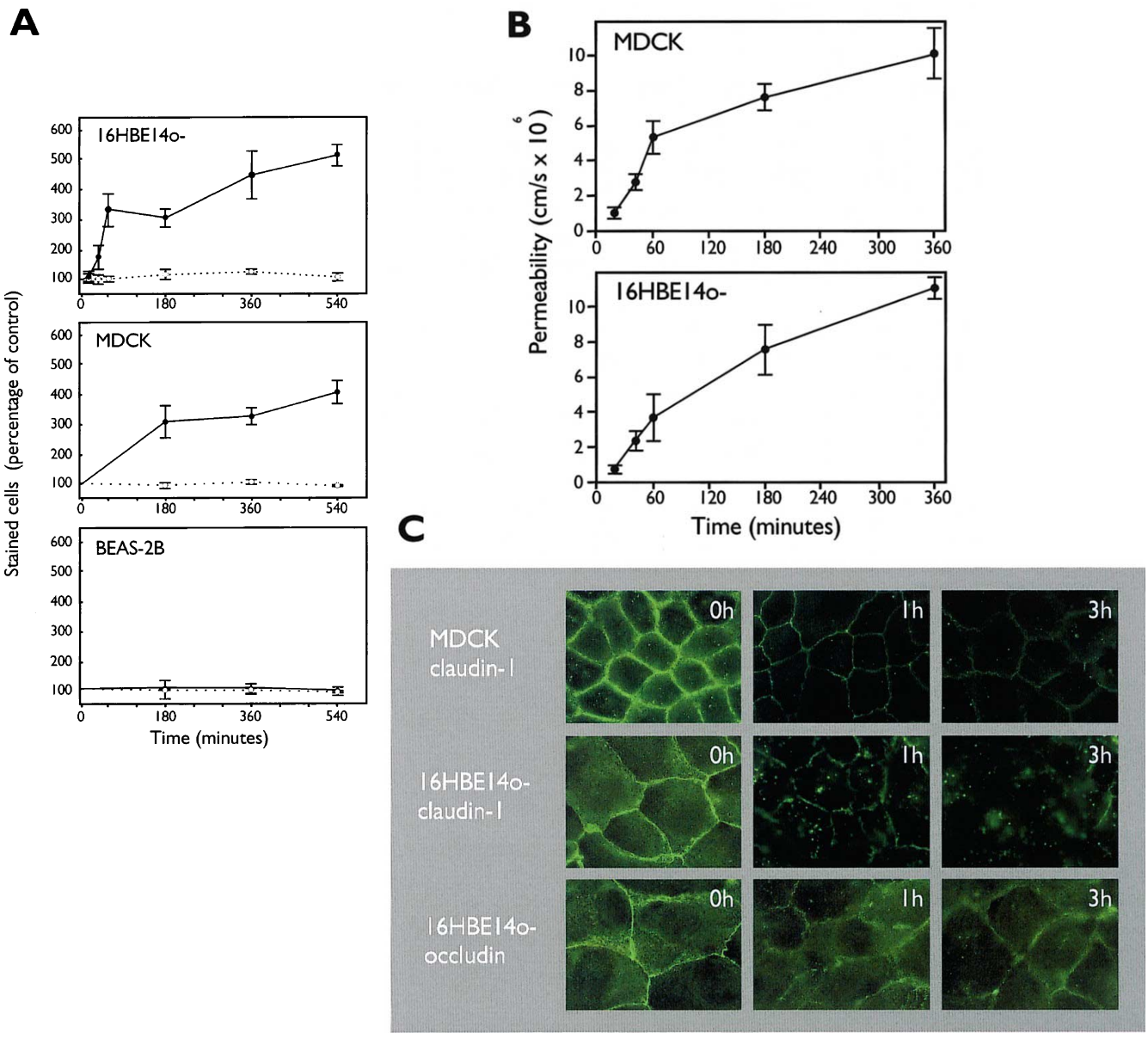

Figure 2. Time-dependent induction of apoptosis in epithelial cell lines by apically applied Der p 1 and its similarity to TJ adhesion protein cleavage and increased paracellular permeability. The development of AV^+ staining (solid lines) and PI^+/AV^+PI^+ staining (dotted lines) in 16HBE14o-, MDCK and BEAS-2B cells treated with Der p 1 in the presence of 0.5 mM GSH is shown in (A). Data are the mean $\pm$ SE mean of three experiments. The increased paracellular permeability of MDCK and 16HBE14o- cell monolayers to mannitol following Der p 1 treatment is shown in (B), with data from eight experiments. Because of the differential sensitivities of MDCK and 16HBE14o- cells to Der p 1, experiments shown in (A) and (B) were conducted with 50 and 500 faecal pellet equivalents, respectively. (C) Representative fluorescent antibody labelling of changes in the staining pattern of claudin-1 (MDCK and 16HBE14o- cells) and occludin (16HBE14o- cells) following treatment of monolayers with allergen under the same conditions as shown in (A) and (B).

respond to Der p 1, being absent in the Der p 1-resistant cell line BEAS-2B unless PMA treated, but constitutively expressed in 16HBE14o- and 1HAEo- cells.

To investigate if the differential expression of TNFα and/or its receptor provided the basis of Der p1 sensitivity, we examined the expression of TNFRI. Transcripts encoding TNF RI were found in all three of the epithelial cell lines, regardless of allergen exposure (Figure 3C) and TNF RI protein was confirmed by immunofluorescence of 16HBE14o- and BEAS-2B cells (Figure 3D). However, TNFα failed to replicate the effect of Der p 1 in 16HBE14o- cells (Figure 3E), suggesting that it did not mediate allergen-induced apoptosis.

Disruption of intercellular adhesions as a trigger for apoptosis

The experiments described above revealed that the increase in paracellular permeability and development of AV^+ staining occurred within 3 h following allergen exposure. To test the possibility that this was because the events were linked, we

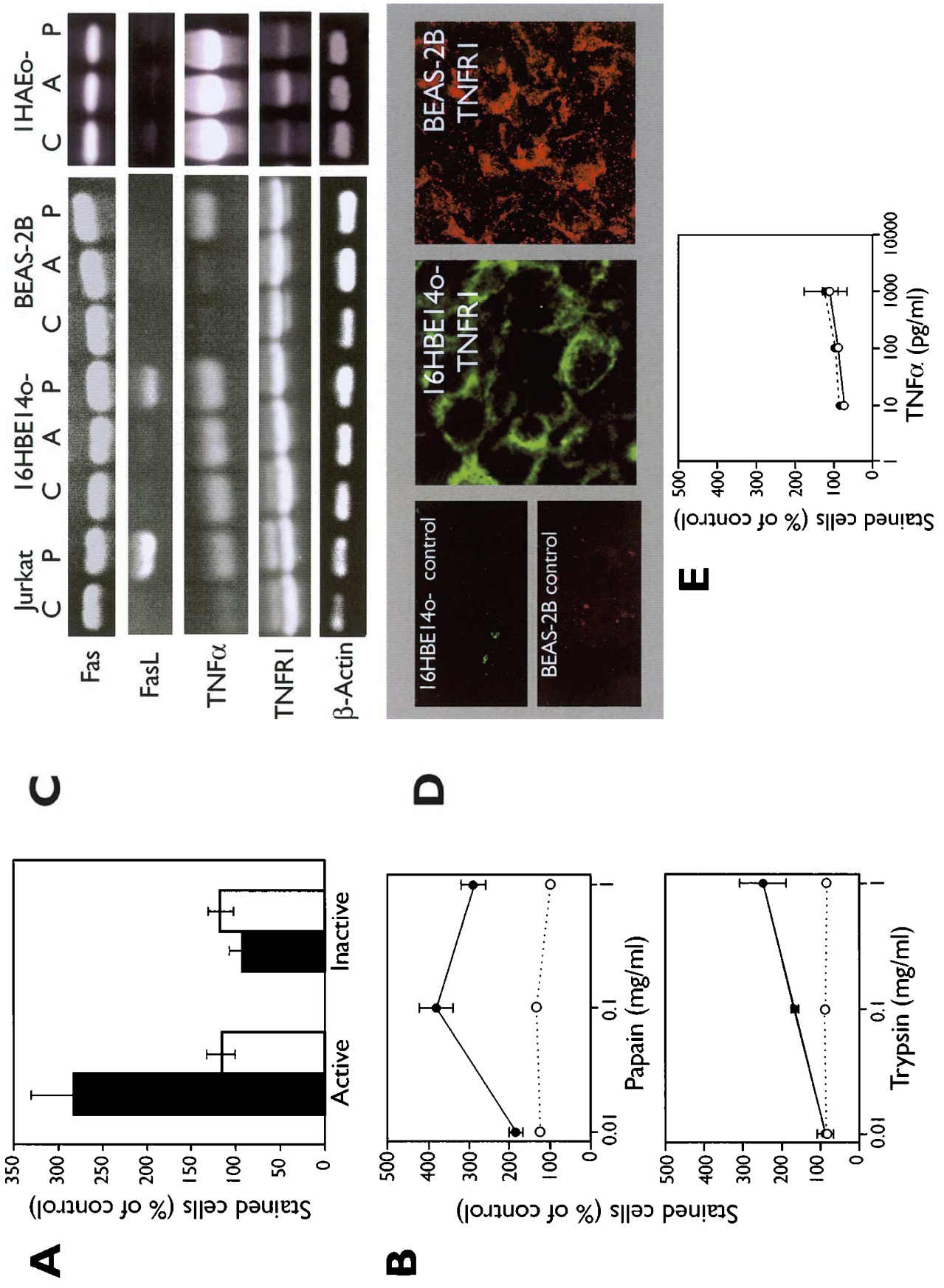
A
Stained cells (% of control)
350
300
250
200
150
100
50
0
Active
Inactive
B
Stained cells (% of control)
500
400
300
200
100
0
0.01
0.1
1
Papain (mg/ml)
Trypsin (mg/ml)
C
Jurkat
C P
16HBE14o-
C A P
BEAS-2B
C A P
1HAEo-
C A P
Fas
FasL
TNFα
TNFR1
β-Actin
D
16HBE14o- control
BEAS-2B control
16HBE14o- TNFR1
BEAS-2B TNFR1
E
Stained cells (% of control)
500
400
300
200
100
0
1
10
100
1000
10000
TNFα (pg/ml)

Figure 3

first compared interepithelial adhesion molecule expression in the cell lines to establish what similarities existed between responding and non-responding cell lines.

One of several differences in cell adhesion between Der p 1-responsive 16HBE14o- cells and Der p 1-non-responsive BEAS-2B cells is the lack of TJs, indicated by the absence of ZO-1 and occludin rings in the latter cell line (Figure 4A). In contrast, MDCK cells, which have well-characterized TJs, exhibited contiguous rings of both proteins (data not shown). Other phenotypic differences in cell adhesion were found. BEAS-2B cells contained transcripts for the desmosomal cadherins Dsc3 and Dsc2 (Figure 4B), but expressed few desmosomes as judged by the weak desmoplakin immunostaining (Figure 4A). In contrast, strong punctate patterns of desmosomal immunostaining were found in 16HBE14o- cells (Figure 4A), similar to those in MDCK cells and normal bronchial epithelium. The major desmosomal cadherins in 16HBE14o- cells appear to be Dsc2, Dsc3 and Dsg2 (Figure 4B). A further significant difference between BEAS-2B and 16HBE14o- cells concerns the expression of classical cadherins. Continuous belts of adherens junctions comprising E-cadherin were evident in 16HBE14o- cells (Figure 4B), similar to those in MDCK cells and in normal airway epithelium (data not shown). Although BEAS-2B stained with a pan-cadherin antibody (Figure 4A), the cadherin detected in BEAS-2B cells was, surprisingly, N- and not E-cadherin (Figure 4B).

However, functional deficiencies in adhesion molecule expression, especially in TJs, are unlikely to explain the differential reactivity to Der p 1. Figure 4A shows that TJ rings are absent in the 1HAEo- cell line, but this actually has a greater sensitivity to Der p 1 than to 16HBE14o- cells that express TJs (Figure 1A). This result would not be expected if TJ proteolysis was the initiator of apoptosis following Der p 1 exposure.

To provide a further test of whether TJs and other lateral cell adhesions are involved in the apoptotic response to Der p 1, we compared the responses of MDCK cells cultured conventionally and in low calcium medium (LCM) (Figure 5). In LCM, cells were unable to assemble peripheral rings of occludin, claudin-1, claudin-2 or ZO-1 into TJs (Figure 5A). Desmosomes, visualized by desmoplakin immunostaining, and adherens junctions, visualized by immunostaining of E-cadherin, were also absent in LCM (Figure 5A). However, LCM treatment did not affect the AV^+ staining response to Der p1 and did not induce PI^+ or combined AV^+PI^+ staining (Figure 5B).

Discussion

These studies provide the first insight into the unusual apoptotic response of epithelial cells to peptidase allergens such as Der p 1. These allergens promote leakiness in epithelial barriers because they cleave the TJ adhesion protein occludin [24]. Although TJs have a potential for bidirectional signalling, the evidence does not support the hypothesis that TJ cleavage activates programmed cell death. Nevertheless, the cleavage of TJs and loss of polarity by the peptidase allergen provide a required remodelling event for eventual cell expulsion from the monolayer. In contrast, the large majority of stimuli that induce apoptosis have no direct effect on intercellular adhesion and there is neither a consensus understanding of how junctional remodelling occurs under such conditions nor agreement on the processing of moribund cells.

In previous work we established that HDM peptidase allergen extracts trigger apoptosis of epithelial cells when made to detach in large numbers from cell monolayers [27]. Whilst it is well known that loss of spatial cues provided by matrix adhesion stimulates apoptosis [4], this would be an unlikely mechanism for the activation of apoptosis by peptidase allergens. When applied apically to epithelial barriers, macromolecules are unlikely to gain easy access to cell–matrix adhesions until TJ cleavage is extensive and paracellular pathways have opened. Experimental evidence shows that Der p 1 does not cross epithelial barriers until TJs are cleaved [24]. As shown here, cells treated with apically applied Der p 1 under less aggressive conditions developed AV^+ staining without expulsion from cell monolayers and there was no obvious concordance between the occurrence of apoptosis in random clusters and the gradual cleavage of TJs that was more uniformly distributed across cell monolayers.

There are some similarities and important differences between the effect of Der p 1 reported here and two other studies where the fates of intercellular junctions were studied in apoptotic epithelial cells. In both of these earlier studies it was observed that cells became apoptotic whilst cell monolayers remained confluent, similar to the effect of Der p 1. Corfe et al. [31] studying the effects of staurosporine in MDCK cells reported that extrusion from monolayers occurred only after cells had become apoptotic and that the extrusion process involved the apparently seamless remodelling of junctions to close the gap between remaining healthy cells. Peralta Soler et al. [32] found that TNFα caused apoptosis in LLC-PK1 cells without loss of monolayer confluence. However, in contrast to Corfe *et al.* it was found

Figure 3. Allergen-induced apoptosis of epithelial cells is dependent on proteolytic activity and does not involve the Fas/Fas ligand death receptor pathway or TNFα/TNF R1 interaction. (A) Apoptosis in MDCK cell monolayers treated for 2 h with 50 faecal pellet equivalents of Der p 1 peptidase activity in the presence of 0.5 mM GSH. Solid bars show cells that stained AV^+, open bars those that stained PI^+/AV^+PI^+. Responses to the catalytically competent and heat-treated allergen are denoted as 'active' and 'inactive', respectively. Data are the mean $\pm$ SE mean of three experiments. (B) Effects of 1-h apical exposure to papain (plus 0.5 mM GSH) and trypsin on MDCK monolayers with data being the mean $\pm$ SE mean of three experiments. In each case, solid lines denote cells with AV^+ staining and the dotted lines the responses for PI^+/AV^+PI^+. (C) Examination by RT-PCR of Fas, Fas ligand (FasL), TNFα and TNF RI in human airway epithelial cells under control conditions ('C') or following treatment with house dust mite allergen (500 faecal pellet equivalents for 3 h) ('A') or phorbol ester (10 ng ml^{-1} for 3 h) ('P'). Jurkat cells were used as a positive control for Fas and Fas L expression. β-Actin was the housekeeping gene. Expression of TNF R1 protein was confirmed by fluorescent antibody labelling of 16HBE14o- or BEAS-2B cells (D). Fluorescent antibody labelling of cells stained with non-immune serum was used to provide control images shown. (E) Treatment of 16HBE14o- cells with TNFα for 1 h had no effect on AV^+ on PI^+/AV^+PI^+ staining.

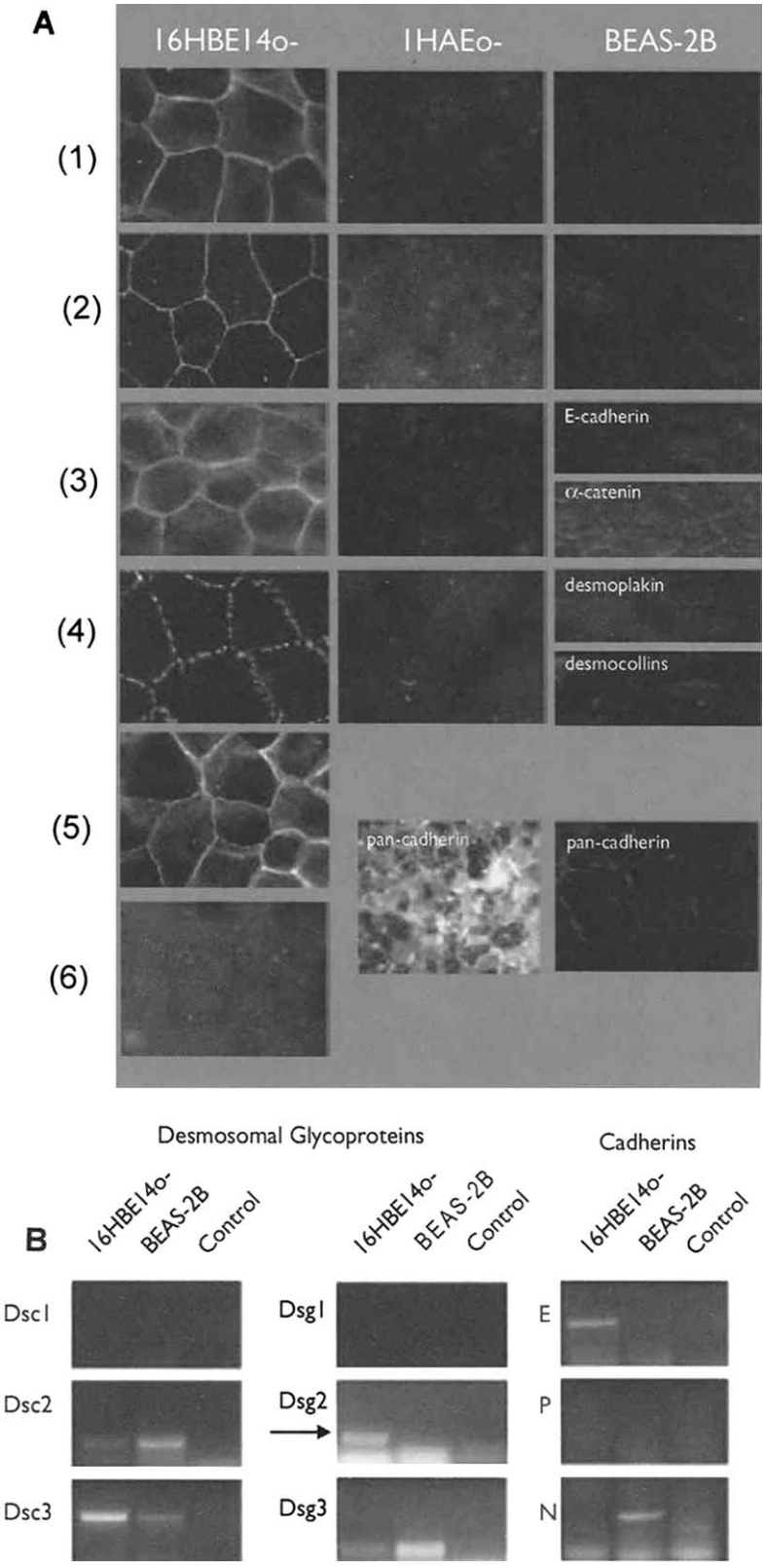

Figure 4. Characterization of intercellular adhesions in airway epithelial cell lines 16HBE14o-, 1HAEo- and BEAS-2B. Representative fluorescent antibody labelling of a range of proteins involved in intercellular adhesion is shown in (A), for (1) occludin, (2) ZO-1, (3) E-cadherin, (4) desmoplakin, (5) claudin-1, (6) claudin-2. The usage of desmosomal glycoproteins and E-, P- an N-cadherin by 16HBE14o- and BEAS-2B cells as determined by RT-PCR is shown in (B). The arrow indicates the Dsg2 amplicon in 16HBE14o- cells.

that as intercellular junctions were remodelled to maintain the epithelial barrier the dying cells were engulfed by their healthy neighbours [32]. Although Der p 1 can induce apoptosis without cells having to exit the monolayer, the overall effects of Der p 1 are more complex and clearly different from agents such as staurosporine and TNFα which perturb the epithelial barrier minimally.

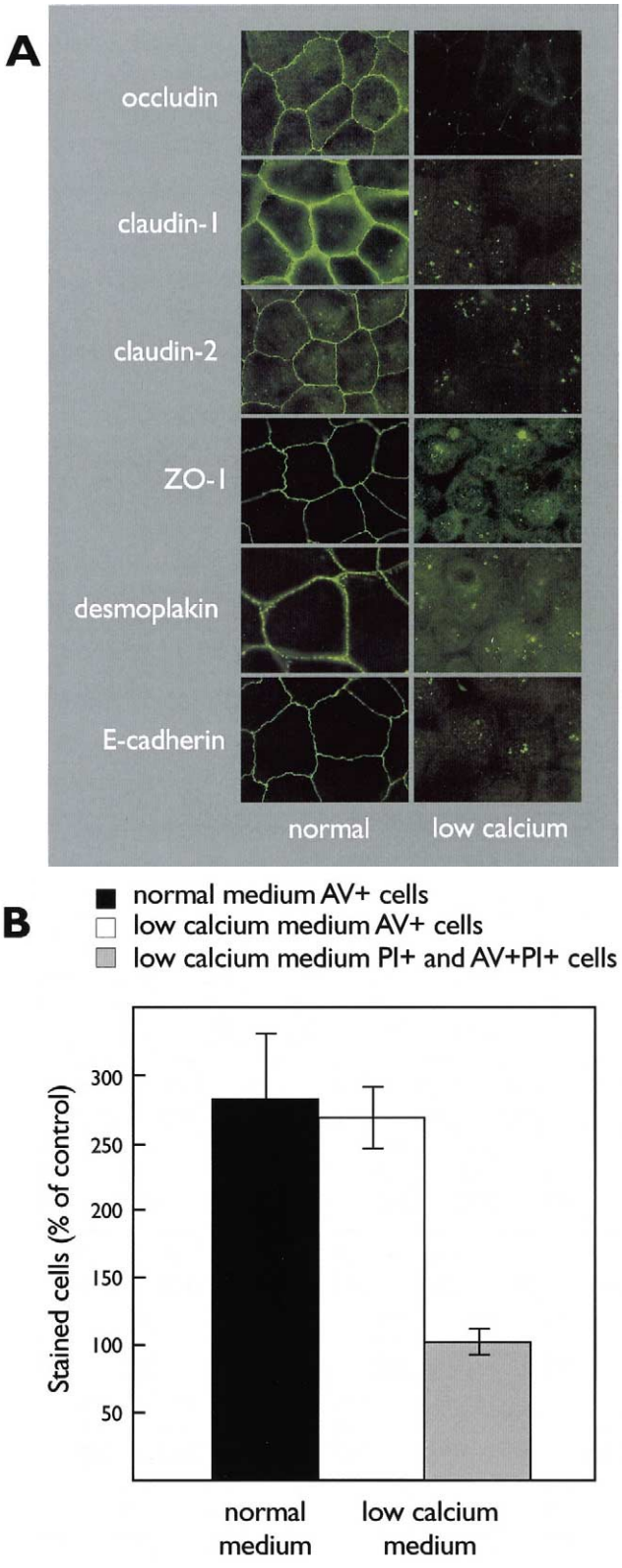

Figure 5

In the present study, we established for the first time that Der p 1 causes loss of claudin-1 from TJ rings. This shows that Der p 1 causes cleavage not only of occludin, a protein intimately involved in TJ permeability control, but also of the claudin family of proteins responsible for the characteristic anastomosing strands of TJs. Whilst actions on claudin-1 and occludin are likely to underpin the increase in epithelial permeability that drives the transepithelial delivery of Der p 1, two significant lines of evidence functionally dissociate TJ cleavage and increased epithelial permeability from the induction of apoptosis. In doing so, this evidence implies that cytoskeletal perturbation per se is insufficient for an apoptotic response to these agents. First, 1HAEo- cells lack TJs but tellingly develop AV^+ staining after treatment with Der p 1. Second, the ability of Der p 1 to induce apoptosis was unaffected in cells where intercellular junction formation had been directly prevented by propagation in LCM. Thus, perturbation of TJs or other lateral adhesions is not an activation signal for apoptosis in this model. However, as described above, the ability of peptidase allergens to cleave TJs and other intercellular junctions provides a convenient means of removing apoptotic cells from the monolayer, albeit at the expense of the integrity of the epithelial barrier.

Extracellular enzymes have been implicated as putative triggers of apoptosis in several other experimental systems. Proteinase-3 and elastase both induce apoptosis in vascular endothelial cells [33], although the events are more protracted than those described here and the response appears driven by outright cell detachment. Pam 212 keratinocytes are spontaneously apoptotic due to the effects of a secreted serine peptidase [34], and granzymes evoke apoptosis of cytotoxic T cell targets following their perforin-dependent translocation to the nucleus of a target cell [35]. Although these studies highlight the ability of peptidases to induce apoptosis, it seems unlikely that a single mechanism underpins the response to these diverse enzymes. How Der p 1 induces apoptosis has proven to be an as yet technically intractable problem, although the evidence presented here shows it to be independent from Fas/FasL and TNF/TNFR I interactions.

The consequences of apoptosis induced by peptidase allergens are unknown. In the airway epithelium, extrusion of dying cells in a way that compromises the epithelial barrier could be a decisive factor in the transepithelial delivery of

Figure 5. TJ cleavage by Der p 1 does not activate apoptosis in epithelial cells. (A) Effect of low calcium medium on the expression of cell adhesions in MDCK cells visualized by fluorescent antibody labelling of TJ proteins (occludin, claudins-1 and -2, and ZO-1), the desmosomal plaque protein, desmoplakin, and E-cadherin of *zonulae adherentes*. (B) Responses of MDCK cells treated apically for 1 h with 50 faecal pellet equivalents of allergen in the presence of 0.5 mM GSH. The black bar shows AV^+ staining of allergen-treated MDCK cells propagated in normal medium. The open bar shows corresponding data for cells propagated in a low calcium medium. Low calcium treatment had no effect on the number of cells staining PI^+ or AV^+PI^+ as indicated by the grey bar. Data are the mean $\pm$ SE of 12 experiments.

allergens [29]. In cells that do not become apoptotic, recovery of TJs from the effects of peptidase allergen exposure is rapidly initiated [24,26] and closure of gaps in the epithelial monolayer with formation of new junctions are likely to restore barrier function. However, outright cell loss in an environment where intercellular adhesion had been compromised by the effects of peptidase allergens would cause a large but highly localized increase in epithelial permeability that might increase the probability of allergen detection by dendritic antigen-presenting cells.

Experimental procedures

Materials

The following were obtained as indicated. Chelex 100 was purchased from Bio-Rad (Hemel Hempstead, UK). Mouse monoclonal TNF RI antibody (clone 16805.21) and reagents for annexin V and propidium iodide staining were from R&D Systems (Abingdon, UK). Tissue culture reagents and murine epidermal growth factor were obtained from Life Technologies (Paisley, UK). Heat-inactivated foetal bovine serum was obtained from TCS Biologicals Ltd (Uckfield, UK). D-[^{14}C]-mannitol was obtained from NEN Du Pont (Brussels, Belgium). TNFα was obtained from Calbiochem CN Biosciences (Nottingham, UK). Spent house dust mite culture medium was obtained from the Commonwealth Serum Laboratories (Parkville, Victoria, Australia). Rabbit polyclonal antibodies to occludin, ZO-1, claudin-1 and -2 were obtained from Zymed, Inc. (San Francisco, CA, USA). Desmoplakin was visualized using mAb 11-5F as previously described [36]. Rabbit anti-pan cadherin and rat E-cadherin mAb (DECMA-1) were purchased from Sigma-Aldrich (Poole, UK). Conjugated secondary antibodies for fluorescent antibody labelling were from Sigma-Aldrich or Chemicon International Ltd (Harrow, UK). Trypsin (type II from porcine pancreas), papain (crystallized, from *Papaya* latex), cholera toxin from *Vibrio cholerae*, soybean trypsin inhibitor cross-linked to 4% beaded agarose, dithiothreitol (DTT), reduced glutathione and *N*-benzoyl-FVR-*p*-nitroanilide were purchased from Sigma-Aldrich. All other laboratory reagents were from Merck Eurolab (Lutterworth, UK) and were of the highest grade obtainable.

Cell culture

16HBE14o-, 1HAEo- (kind gifts of Professor Dieter Gruenert, University of Vermont, USA) and BEAS-2B (a kind gift of Dr Curtis Harris, National Cancer Institute, Bethesda, MD, USA) are cell lines established from human bronchial epithelium by SV40 transformation [37–39] and were cultured as described [29,30]. Properties of these cell lines are catalogued elsewhere [29,30]. Madin–Darby canine kidney (MDCK) cells, propagated as described elsewhere [30], were studied for comparison. Jurkat cells, cultured in RPMI 1640 containing 10% (v/v) foetal bovine serum supplemented with L-glutamine and antibiotics, were used as a positive control in RT-PCR experiments.

Low calcium treatment

MDCK cells were seeded into calcium-free EMEM containing L-glutamine and antibiotics. The medium was modified to contain high glucose (4.5 g l^{-1}). Foetal bovine serum was depleted of divalent cations by batchwise treatment with Chelex 100 resin according to manufacturer's instructions. To optimize cell growth, this medium was supplemented with hydrocortisone (0.5 μg ml^{-1}), cholera toxin (10^{-10} M) and EGF (10 ng ml^{-1}) as described [40].

Paracellular permeability measurements

Paracellular permeability to mannitol was measured at selected times following apical application of allergen to epithelial monolayers as detailed elsewhere [29]. For permeability studies, cells were propagated in Transwells with 0.4 μm pore diameter polycarbonate membranes (Corning-Costar Ltd, High Wycombe, UK).

Preparation of Der p 1 fractions

Faecal-pellet enriched culture medium in which *Dermatophagoides pteronyssinus* had been cultured was fractionated by ammonium sulphate precipitation as described previously [27]. Der p 1 was purified from the cysteine peptidase fraction as described elsewhere [24]. To ensure maximum catalytic activity of Der p 1, studies were conducted in the presence of 0.5 mM GSH. Our previous studies have shown that under these conditions the reducing agent has no observable effect on epithelial permeability [26,25,27]. Allergen preparations were free of contaminant serine peptidase activity according to measurements of enzymatic activity before and after extensive batchwise treatment with a suspension of soybean trypsin inhibitor coupled to agarose gel. Heat-inactivated allergen was prepared by boiling for 10 min.

The peptidase activity of allergen extracts was standardized against the proteolytic activity contained in defined numbers of faecal pellets that had been 'aged' for 8 months at room temperature and humidity as described previously [24]. Enzymatic activity of Der p 1 was measured spectrophotometrically using *N*-benzoyl-FVR-*p*-nitroanilide (single amino acid letter notation). Briefly, assays were performed at 37°C in a final volume of 200 μl phosphate-buffered saline (PBS) and the V_{max} measured. Substrate concentration was 0.63 mM and the reaction mixture contained 0.6 mM DTT with suitable dilutions of the peptidase allergen. Calibrated lots of allergen were then used in experiments in amounts that could be standardized to equivalent numbers of faecal pellets.

RT-PCR

Epithelial cells were grown to confluence in 25 cm^2 flasks and culture media replaced with EMEM supplemented with antibiotics, glutamine and 10% v/v Nu-serum IV (Stratech Scientific Ltd, Luton, UK). Cells were treated with either cysteine peptidase allergen (100 faecal pellet equivalents) for 24 h or 10 ng ml^{-1} phorbol 12-myristate 13-acetate (PMA) as a control inducing agent for 3 h. Jurkat cells were grown to a density of 10^6 cells ml^{-1} in 10 ml medium and treated for 3 h with 10 ng ml^{-1} PMA and 1 μM ionomycin. Total cellular RNA was extracted using RNeasy kits (QIAgen, Crawley, UK).

First strand cDNAs were synthesized by incubating RNA (5 μg) for 90 min at 37°C with M-MLV reverse transcriptase (Life Technologies) using poly d(T)$_{12-18}$ as primer. After cDNAs had been denatured for 5 min at 94°C, PCR was performed with 12.5 pmol each of the 5′ and 3′ primers, 1 μl cDNA, 1.25 u HotStarTaq DNA polymerase (QIAgen) and 2.5 nmol each of dNTPs. After initial incubation at 94°C for 15 min, amplification was carried out for 35 cycles with denaturation at 94°C for 1 min, annealing at optimal temperatures calculated for each primer pair for 1 min and with extension at 72°C for 2 min. Amplified products were analysed by electrophoresis on 1.5% w/v agarose gels according to conventional technique. Primer pairs for PCR (5′-sense-3′:5′-antisense-3′) were: Fas ATGCTGGGCATCTGGACCCTCCTA:TCTGCACTTGGTATTCTGGGTCCG; FasL GTCCCAGAAGGCCTGGTCAAAGG:TTGCAAGATTGACCCCGGAAGTAT; TNFα CGAGTGACAAGCCTGTAGCC:CATACCAGGGCTTGGCCTCA;TNF R1 ATTTGCTGTACCAAGTGCCACAAAGGAACC:GTCGATTTCCCACAAACAATGGAGTAGAGC; human Dsc1 TTGGATACAAAGCACTGGACC:CCAGAAAGAATTGAAAAGGTGG; human Dsc2 ATGACTGCACACATCGTGTAGATCC:TGTCCTCCTTTCACCATTTCG; human

Dsc3 GTGCGACTTCAAGGAGTACAGG:TCCAGGGTATGATGATCCC; human Dsg1 GGTGGGGCAGATG:TCCCACTTTATCATTTGATCCC; human Dsg2: ACCACCTGAAGACAAGGTGG:TGGTTTCAGTGGTCATGATAGC; human Dsg3 GATAGTGACAGAAAGGGTGA:CTACAATTTGAATAGTACTTG; human E-cadherin TTTGTACAGATGGGGTCTTGC:CAAGCCCACTTTTCATAGTTCC; human P-cadherin CCTACTCCCAGAAGATGACACC:GAACACCAAGAGGGTGTCGT; human N-cadherin TAGTCACCGTGGTCAAACCA:CTATTTTTAGCCAATTGGCAGG and β-actin GGCTCTCTTCCAGCCTTCCTTCCT:CACAGAGTAACTTGCGCTCAGGAGG.

Quantification of apoptosis by annexin V staining

Cells were cultured to confluence in eight-well Nunc Lab-Tek® chamber slides (Life Technologies). Allergen treatment was performed in EMEM culture medium in which FBS was replaced by 10% v/v Nu Serum IV. In all experiments, allergen was applied to the apical surface of epithelial monolayers. After treatment, cells were washed twice with ice-cold PBS and staining with FITC-conjugated annexin V (2.5 $\mu g\ ml^{-1}$) and propidium iodide (5 $\mu g\ ml^{-1}$) performed for 15 min in pH 7.4 binding buffer (composition, mM: HEPES 10, NaCl 150, KCl 5, $MgCl_2$ 1 and $CaCl_2$ 1.8) under subdued illumination. The staining solution was removed and cells washed twice with binding buffer. Enumeration of labelled cells was performed by epifluorescence microscopy (Zeiss Axiovert 10, Carl Zeiss Ltd, Welwyn Garden City, UK). Cells were declared apoptotic if they stained with annexin V alone (AV^+). Cells staining with propidium iodide (PI^+) were declared necrotic. Dual-labelled cells (AV^+PI^+) were not included in the counts of apoptotic cells. For the purposes of data display, numbers of PI^+ and AV^+PI^+ cells have been aggregated.

Fluorescent antibody staining

Cells were fixed in methanol and fluorescent antibody labelling was performed according to conventional procedures. Secondary antibodies were labelled with DTAF, FITC or TRITC.

Fluorescence microscopy was performed using a Zeiss Axiovert 10 microscope with digital image capture to a Coolsnap 12-bit, cooled CCD camera (Imaging Associates Ltd, Thame, UK). Confocal microscopy was performed using Zeiss LSM410 and LSM510 microscopes. Image processing and analysis was performed using software suites for IDL (Research Systems, Inc., Boulder, CO, USA) running on SGI Indigo2 and Indy workstations (Silicon Graphics, Reading, UK).

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