Polarized fibronectin secretion induced by adenosine regulates bacterial–epithelial interaction in human intestinal epithelial cells

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Fibronectin (FN) is a multifunctional protein that plays important roles in many biological processes including cell adhesion and migration, wound healing and inflammation. Cellular FNs are produced by a wide variety of cell types including epithelial cells, which secrete them and often organize them into extensive extracellular matrices at their basal surface. However, regulation of FN synthesis and the polarity of FN secretion by intestinal epithelial cells have not been investigated. In the present study we investigated the role of adenosine, whose levels are up-regulated during inflammation, in modulating FN synthesis, the polarity of FN secretion and the downstream effects of the secreted FN. Polarized monolayers of T84 cells were used as an intestinal epithelial model. Adenosine added to either the apical or basolateral aspect of the cells led to a time- and dose-dependent accumulation of FN in the culture supernatants, polarized to the apical compartment and reached maximal levels 24 h after apical or basolateral addition of adenosine. Confocal microscopy confirmed that FN localized to the apical domain of model intestinal

epithelial cells stimulated with apical or basolateral adenosine. The induction of FN was significantly down-regulated in response to the adenosine receptor antagonist alloxazine and was inhibited by cycloheximide. Moreover, adenosine increased FN promoter activity (3.5-fold compared with unstimulated controls) indicating that FN induction is, in part, transcriptionally regulated. Interestingly, we demonstrated that adenosine, as well as apical FN, significantly enhanced the adherence and invasion of *Salmonella typhimurium* into cultured epithelial cells. In summary, we have shown for the first time that FN, a classic extracellular matrix protein, is secreted into the apical compartment of epithelial cells in response to adenosine. FN may be a critical host factor that modulates adherence and invasion of bacteria, thus playing a key role in mucosal immune responses during inflammation.

Key words: adenosine, extracellular matrix, fibronectin, inflammation, polarized cell, T84 cell.

INTRODUCTION

Epithelial cells receive cues from the environment through soluble growth factors, luminal bacteria, viruses and insoluble extracellular matrix proteins, form a barrier to a range of noxious substances and are characterized, in part, by their ability to sort and vectorially secrete proteins. Fibronectins (FNs) are ubiquitous multifunctional glycoproteins found in most body fluids, in the extracellular matrix and on cell surfaces, and have binding sites for different cells and molecules, including heparin, DNA, collagen, actin and fibrin, as well as to bacteria and cell surfaces [1]. It is also one of the many matrix proteins deposited on the surface of implanted biomaterials that promotes cell adhesion, migration and cytoskeletal organization, thereby influencing cellular proliferation and differentiation [2–4]. FN exists in two forms, cellular FN and plasma FN. Cellular FN is synthesized by many different cell types, including fibroblasts, chondrocytes, endothelial cells and macrophages, as well as by certain epithelial cells. Cells assemble FN into a fibrillar form that accumulates at their basal cell surface. FN matrix formation is initiated by FN binding to cell surface receptors, followed by assembly and reorganization of the cell-surface-associated FN into fibrils [5]. FN has been shown to contribute to the inflammatory process by increasing chemotaxis and altering the phenotype of leucocytes [6]. In addition, it can recruit and cause fibroblast proliferation, which is involved in tissue repair and fibrosis in diseases such

as inflammatory bowel disease [7]. FN is highly expressed in inflamed tissues and has been shown to be one of the acute-phase proteins in rats, mice and humans [8–10]. However, the regulation and polarity of FN secretion in epithelial cells is not known.

Adenosine is an endogenous primordial signalling molecule that has been established as an important modulator of physiological responses in all mammalian tissues. It is produced during normal cellular metabolism, intestinal ischaemia and inflammation, and adenosine and its precursors are released from damaged cells and discharged from mast cells and platelets [11–13]. A series of reports have appreciated that adenosine has a plethora of biological actions on a large variety of cell types, tissues, organs and physiological processes, and can modulate various functions of cells involved in inflammatory responses [14,15]. Adenosine concentration increases during ischaemia as a result of ATP utilization [16], and has been shown to be increased from less than 300 nM to 500–600 nM in inflammatory lesions [17]. We have previously shown that adenosine induces apically polarized secretion of IL-6 (interleukin-6), a classical pro-inflammatory cytokine, in intestinal epithelial cells, and IL-6 in turn modulates neutrophil function [21]. It has also been shown that adenosine and an adenosine-receptor agonist affect the adherence of neutrophils to vascular endothelial cells, suggesting that adenosine may contribute to the regulation of adhesive interactions of immunocompetent cells to other cell types [17]. In the present study we examined the effect of adenosine in modulating FN

Abbreviations used: CFU, colony-forming units; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FN, fibronectin; HBSS, Hanks balanced salt solution; IL, interleukin; LB, Luria–Bertani.

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synthesis, the polarity of FN secretion from intestinal epithelial cells and the downstream effects of the secreted FN.

Previous findings have led to the understanding that intestinal epithelial cells play a key role in orchestrating the inflammatory response to surface-attached or invading bacteria [19,20]. *Salmonella typhimurium* actively contributes to the generation of a variety of pro-inflammatory signals via the function of the so-called Inv/Spa/Prg Type III secretion system [18]. However, not much is known about the host factors that modulate the adherence and invasion of *S. typhimurium* in epithelial cells. We therefore investigated whether the expression of FN plays a role in *S. typhimurium* adherence and invasion of the human colonic epithelial cell line T84.

EXPERIMENTAL

Reagents

All tissue culture supplies were obtained from Invitrogen Life Technologies (Grand Island, NY, U.S.A.). Anti-FN antibody was obtained from Abcam (Cambridge, MA, U.S.A.), and anti-rabbit secondary antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Human plasma-derived FN and alloxazine {benzo[g]pteridine-2,4(1*H*,3*H*)-dione} were purchased from Sigma (St. Louis, MO, U.S.A.). Adenosine was from Calbiochem (San Diego, CA, U.S.A.). Reagents for SDS/PAGE and nitrocellulose membranes (0.45- μ m pores) were from Bio-Rad (Hercules, CA, U.S.A.). FITC-labelled goat anti-rabbit antibody and horseradish-peroxidase-conjugated Ig were obtained from Jackson ImmunoResearch Laboratory (West Grove, PA, U.S.A.) and Amersham Biosciences (Piscataway, NJ, U.S.A.) respectively.

Cell culture

Human colonic epithelial cells, T84 cells and rat intestinal epithelial cells, IEC-6 cells (A.T.C.C., Manassas, VA, U.S.A.) were grown and maintained in culture in DMEM (Dulbecco's modified Eagle's medium)/Ham's F-12 medium (1:1) supplemented with 10% FBS (fetal bovine serum), 100 units/ml penicillin and 100 μ g/ml streptomycin as described previously [21]. Confluent stock monolayers were subcultured by trypsinization. Experiments were done on cells plated for 7–8 days on permeable supports of 0.33 cm² or 4.5 cm² (inserts). Inserts with rat tail collagen-coated polycarbonate membrane filter (0.4- μ m pore size; Costar, Cambridge, MA, U.S.A.) rested in wells containing medium until steady-state resistance was achieved. This permits apical and basolateral membranes to be separately interfaced with apical and basolateral buffer. COS-7 cells (A.T.C.C.) were grown in DMEM containing 10% FBS supplemented with penicillin and streptomycin.

Western blot analysis

Filter-grown cells were either serum-starved (IEC-6 cells) or starved in 0.5% serum (T84 cells) overnight and then treated apically or basolaterally with adenosine (0–100 μ M) for indicated time points in 0.5% FBS. The transepithelial resistance was not affected under these conditions. The conditioned medium was collected as described previously [21] and the cells were lysed in lysis buffer [PBS containing 1% Triton X-100 and 1% Nonidet P40 (v/v), CompleteTM protease inhibitor mixture (Roche Applied Science Indianapolis, IN, U.S.A.), EDTA, SDS, sodium orthovanadate and NaF], and the total protein concentration of all samples was measured by the Lowry method using protein assay reagent (Bio-Rad). SDS/PAGE of purified FNs were

performed using 7.5% polyacrylamide gels (Bio-Rad) under reducing conditions. The separated proteins were transferred on to a nitrocellulose membrane that was blocked at 22 °C for 1 h in Tris-buffered saline with 0.2% Tween 20 (TBS-T) containing 6% skimmed milk and probed with primary antibody against human plasma FN (1:3000) diluted in TBS-T. The membranes were then incubated for 1 h with anti-rabbit secondary antibody (1:5000). The specific bands of target proteins were visualized by Western Lightning Chemiluminescence Reagent plus (PerkinElmer, Boston, MA, U.S.A.) according to the manufacturer's instructions and were developed on an X-ray film (Denville Scientific, Metuchen, NJ, U.S.A.). Western blots were quantified using Fluorchem (camera imaging densitometer, model 8300; Alpha Innotech Corp, San Leandro, CA, U.S.A.).

Transfection and luciferase assays

For luciferase assays, COS-7 cells were transiently transfected using Lipofectamine2000 (Invitrogen Life Technologies) as described previously [21]. Briefly, 24 h prior to transfection, cells were plated at a density of 2×10^5 cells/well. The next day, cells were transfected with 2.5 μ g/well of the specific luciferase construct (FN-Luc) along with 5 ng/well SV40-RL (Promega) as an internal control for transfection efficiency. After 48 h, cells were washed with PBS and starved in 0.5% serum overnight. Subsequently, cells were stimulated with adenosine (100 μ M) or vehicle for 8, 16 or 24 h. Cells were then lysed and luciferase activity was determined using the Promega Dual Luciferase Reporter Assay according to the manufacturer's instructions. Luciferase activities were normalized to the *Renilla* activities.

Confocal microscopy

Confocal microscopy was performed as described previously [22]. Briefly, monolayers of T84 cells were washed in HBSS (Hanks balanced salt solution) and fixed with 3.7% formaldehyde for 20 min. Subsequently, the cells were permeabilized with 0.5% Triton X-100 for 40 min at 25 °C and then blocked with 2% BSA. Cells were then incubated overnight with respective antibodies diluted in PBS containing 2% BSA (1:100). The cells were washed with HBSS and then incubated with appropriate FITC-labelled secondary antibody diluted at 1:400 (Jackson ImmunoResearch Laboratories). Monolayers were also counterstained with rhodamine/phalloidin to visualize actin. Monolayers, mounted in *p*-phenylenediamine glycerol (1:1), were analysed by confocal microscopy (Zeiss dual laser confocal microscope; Carl Zeiss Inc., Thornwood, NY, U.S.A.).

Bacterial strain and growth conditions

S. typhimurium SL 3201 (wild-type strain) were grown in a modified LB (Luria–Bertani) broth [1% bacto-tryptone (Difco)] as described in [23]. MacConkey agar (Difco) was prepared according to the manufacturer's instructions. Non-agitated micro-aerophilic bacterial cultures were prepared by inoculating 10 ml of LB with 0.01 ml of a stationary-phase culture, followed by overnight incubation (approx. 18 h) at 37 °C. Bacteria from such cultures were in the late exponential phase of growth and correlated to $(5-7) \times 10^8$ CFU (colony-forming units)/ml routinely [23].

S. typhimurium adhesion/invasion into T84 intestinal epithelial cell monolayers

A standard gentamicin protection assay was used to study bacterial invasion into model colonic epithelial cells [23]. Briefly, semi-confluent monolayers of T84 cells grown in 12-well plates were

Figure 1 Adenosine induces apically polarized secretion of FN

T84 monolayers grown in DMEM supplemented with 10 % FBS were serum starved for 24 h in 0.5 % FBS. Adenosine (Ado; 100 μ M) was then added to the apical (Ap) or basolateral (Bl) compartment. After an incubation period of 24 h, culture media in the apical or basolateral compartments were collected and processed for Western blotting to determine FN secretion. Protein bands in the autoradiogram shown in (A) were quantified by scanning densitometry, and densities relative to vehicle-treated controls are shown in the histogram to illustrate the alterations in band intensities. The data are representative of three separate experiments. (B) Confocal imaging of adenosine-induced FN in intestinal epithelial monolayer. Serum starved T84 cells (0.5 % FBS, 24 h) were stimulated with apical or basolateral adenosine (100 μ M) for 24 h, after which they were fixed and stained with anti-FN antibody followed by FITC-conjugated secondary antibody and counterstained with rhodamine/phalloidin, as described in the Experimental section. Vertical sections were taken off the monolayers to define the top (0 μ m) and the bottom of the monolayer (18–20 μ m). Shown here are 'en face' (x - y) images taken at the level of apical (1.2 μ m above the level of tight junction), mid- (6 μ m below the level of tight junction) and basal (18–20 μ m, at the level of the stress fibres) pole of the epithelial monolayer.

either stimulated with adenosine (100 μ M) or vehicle for 24 h and then washed three times with HBSS (Ca^{2+} and Mg^{2+} , with 10 mM Hepes, pH 7.4)] or were co-incubated with FN (12 μ g/ml; apical) and bacteria. Infection was performed for 1 h at 37 °C. Non-adherent bacteria were washed off by thoroughly washing cells with HBSS. Cell-associated salmonellae, that represent populations of bacteria attached to and/or internalized into the T84 monolayers, were then released by incubation with 0.1 ml of 1 % Triton X-100. For determining internalized bacteria, cells were further incubated with 1 ml of fresh medium containing 500 μ g/ml gentamicin to kill residual extracellular bacteria. After a further 2 h incubation period at 37 °C, infected cells were lysed by the addition of 1 % Triton X-100 for 90 min. For both cell-associated and internalized bacteria, 0.9 ml of LB medium was then added, and each sample was vigorously mixed and quantified by plating in serial dilutions for CFU on MacConkey agar medium. The invasive efficiency was expressed as the number of viable internalized bacteria by counting the CFU.

Statistical analysis

Statistical significance between the individual groups was analysed using the unpaired Student's t test with a threshold of $P < 0.05$.

RESULTS

Adenosine induces polarized FN secretion

Polarized epithelial cells have the ability to vectorially secrete proteins. To determine if adenosine modulates FN secretion and whether this modulation is polarized, confluent monolayers of intestinal epithelial T84 and IEC-6 cells, grown in tissue culture inserts (Corning, Acton, MA, U.S.A.), were stimulated via the apical or basal surface with adenosine (0–100 μ M), and conditioned media were analysed by Western blot analysis to examine the steady-state level of FN protein. As seen in Figure 1(A), FN

Figure 2 Adenosine induces FN secretion in IEC-6 cells

IEC-6 monolayers were stimulated with apical or basolateral adenosine (Ado; 100 μ M). Conditioned media were collected at indicated times after adenosine treatment and FN was measured as described in the Experimental section. Protein bands in the autoradiograms were quantified by scanning densitometry and histograms of the densities relative to vehicle-treated controls are shown to illustrate the alterations in band intensities. The data are representative of the responses observed in two separate experiments carried out in duplicate.

levels in the T84 culture media was significantly increased at 24 h following the addition of adenosine, but the secretory response to adenosine stimulation differed depending on which surface FN was measured. The secretion of FN into the basolateral medium was increased only mildly following both apical and basal stimulation by adenosine. In contrast, apical secretion of FN increased significantly after apical or basal adenosine stimulation. Adenosine (100 μ M) added to the apical or basolateral compartment for 24 h induced approx. 10- and 12-fold increases respectively in FN levels compared with T84 cells treated with vehicle alone. To further localize FN upon adenosine stimulation in confluent T84 monolayers, we used immunofluorescence staining. Cells were fixed with paraformaldehyde and detected using an antibody against human FN. As seen in Figure 1(B), FN was detected in the apical cell surface of confluent T84 cells stimulated with apical or basolateral adenosine. Control monolayers had no detectable intracellular FN staining. In order to validate the result obtained using transformed colonic epithelial cell line T84, we further investigated the effect of adenosine in IEC-6 cells. IEC-6 cells are primary rat intestinal epithelial cells and are similar both functionally and morphologically to human crypt epithelial cells [24]. As shown in Figure 2, apical or basolateral adenosine induced the secretion of FN into the apical media, as seen in T84 cells. This increase in FN secretion peaked at 48 h.

Time course of FN induction by adenosine

The time course of FN induction was performed in the presence of 100 μ M adenosine. After 24 h in medium containing 0.5% FBS, adenosine was added to the cultures, apically or basolaterally, and conditioned media were collected after 8, 16, 24 and 48 h. Western blot analysis was used to follow the FN levels as described in the Experimental section. Densitometric quantification of the FN protein bands demonstrated that significant FN secretion into the apical media occurred 8–16 h after exposure to adenosine, and peaked after 24 h following apical (approx. 7-fold compared with control) and basolateral (14-fold compared with control) adenosine stimulation (Figure 3).

Figure 3 Time course of FN levels after adenosine stimulation

T84 monolayers were serum starved in 0.5% FBS. After 24 h, cells were stimulated with adenosine (apical or basolateral; Ado, 100 μ M), culture media were collected at 8, 16, 24 and 48 h, and Western blotting was performed as described in the Experimental section. Autoradiographs were quantified by densitometric image scanning and are expressed in the histograms as fold-increase over vehicle-stimulated controls. Data represent the responses observed in two separate experiments.

Adenosine induces FN secretion in a dose-dependent fashion

To explore further the relationship between adenosine doses and FN induction, FN levels in the medium of T84 cell cultures was measured 24 h after increasing doses of apical and basolateral adenosine (0–100 μ M) treatment. FN secretion into the media increased in a dose-dependent fashion, and increased by 9.6-, 9.8-, 11.1- and 12.3-fold compared with controls following apical treatment with 5, 10, 50 and 100 μ M adenosine respectively (Figure 4A). In cells treated with basolateral adenosine, a similar dose-dependent increase in FN secretion was observed where the FN levels increased by 5.9, 2.4-, 7.4-, 10.1- and 6.5-fold with 5, 10, 50 and 100 μ M adenosine respectively (Figure 4B).

Adenosine-induced FN induction is mediated by the A2b receptor

Adenosine mediates its physiological effects via interaction with one or more of its four known receptors: A1, A2a, A2b and A3. However, T84 cells have been shown to possess only the A2b subtype, and signalling through the A2b receptor is mediated by cAMP and protein kinase A [25]. In order to determine if adenosine-induced FN synthesis is mediated by the A2b receptor, T84 cells were incubated with alloxazine (50 μ M), an adenosine receptor antagonist along with 100 μ M adenosine. Culture medium was then assessed for immunoreactive FN protein using Western blotting. As seen in Figure 5 (lanes 2 and 3), FN secretion induced by apical (lane 6) or basolateral (lane 7) treatment of T84 cells with adenosine for 24 h was significantly suppressed when cells were also co-incubated with alloxazine, suggesting that adenosine-stimulated FN secretion was mediated via activation of A2b receptors. We further investigated if FN induction by adenosine involved *de novo* protein synthesis. As seen, pre-treatment of T84 cell monolayers with the protein synthesis inhibitor cycloheximide (50 μ M, 30 min pretreatment) abolished the adenosine-mediated increase in FN protein secretion (Figure 5, lanes 4 and 5), implying that the induction of FN by adenosine may require protein synthesis.

Figure 4 Dose-response of adenosine-induced FN secretion

T84 cells growing in monolayer were treated with increasing doses of 0.005, 0.01, 0.05 or 0.1 μ M of adenosine (Ado), at either the apical (A) or basolateral (B) side, and culture media were collected after 24 h. Western blotting was performed as described in the Experimental section. Autoradiographs were quantified by densitometric image scanning and expressed in the histograms as fold-increase over vehicle-stimulated controls. Data presented are representative of the responses observed in three separate experiments.

Figure 5 Effects of A2b receptor inhibitor and cycloheximide on adenosine-induced FN

T84 cells were pretreated with cycloheximide (50 μ M) for 30 min before the addition of apical or basolateral adenosine (100 μ M). Alloxazine (50 μ M) was added along with apical or basolateral adenosine. After 24 h, conditioned media were collected and FN protein levels were analysed by Western blot analysis as described in the Experimental section. The blot is representative of two separate experiments done in duplicate.

Adenosine activation of fibronectin promoter activity in intestinal epithelial cells

On the basis of our results that cycloheximide inhibited the synthesis of adenosine-induced FN, we next determined whether adenosine was able to induce FN promoter activity. We performed a transient transfection assay in the COS-7 cell line with a luciferase reporter gene linked to the regulatory region of human FN gene (hFN-LUC), along with *Renilla* reporter vector serving as an internal control. The FN promoter construct contains approx. 1200 bp of the 5'-flanking region of the human FN gene isolated from the human fibrosarcoma cell line HT-1080 and contains several regulatory elements, such as three cAMP response elements, located at -415 bp, 260 bp and 170 bp, and an Sp-1 site at 102 bp from the transcription start site. COS-7 cells possess A2b receptors and were used in view of the difficulty in transfecting T84 cells [21]. After transfection (48 h), cells were stimulated with 100 μ M adenosine for 8, 16 or 24 h. Normalized

luciferase activity indicated that adenosine induced an approx. 3.2-fold increase in relative luciferase activity after 24-h adenosine stimulation compared with cells stimulated with vehicle alone, suggesting that adenosine induces FN secretion at least in part through transcriptional regulation (results not shown). We also confirmed these results using CHO (Chinese-hamster ovary) cells co-transfected with A2b receptor and the hFN-LUC construct (results not shown).

Adenosine and fibronectin promotes *S. typhimurium* adherence and invasion of cultured intestinal epithelial cells

Many pathogenic bacteria, including *S. typhimurium*, have been demonstrated to invade eukaryotic cells *in vitro*. Migration through tissue barriers is a major task for a bacterial pathogen in its dissemination within the host, and FN has been shown to play an important role in bacterial adhesion and invasion [26,27]. To investigate whether adenosine or FN released preferentially into the apical compartment of model intestinal epithelia aid in *S. typhimurium* uptake, we employed the gentamycin protection assay, which is a standard invasion assay used extensively to analyse cellular invasion by several bacterial species. Epithelial cells were incubated with vehicle or adenosine (100 μ M) for 24 h. Cells were subsequently infected with *S. typhimurium* and the adherence/invasion assay was performed as described in the Experimental section. The number of adhering *S. typhimurium* in T84 cells exposed to adenosine was significantly increased compared with vehicle-treated monolayer [adenosine/*S. typhimurium*, $(23.8 \pm 5) \times 10^6$ CFU; vehicle/*S. typhimurium* $(6 \pm 2.4) \times 10^6$ CFU] (Figure 6A). Additionally, our results demonstrate that *S. typhimurium* invaded cultured T84 cells [Figure 6B, $(0.3 \pm 0.02) \times 10^6$ CFU per insert, or approx. 1% of total bacteria applied to the monolayer]. Treatment of epithelial cells with adenosine for 24 h significantly increased bacterial invasion of intestinal epithelial cells with $(0.7 \pm 0.08) \times 10^6$ CFU recovered from gentamycin-treated monolayer (Figure 6). Similar results were obtained when monolayers were infected with *S. typhimurium* in the presence of FN (12 μ g/ml; apical). In this case, FN was more potent compared with adenosine and up-regulated bacterial adherence and invasion by 12-fold (Figure 6C) and 4-fold (Figure 6D) respectively. Furthermore, using confocal imaging, we demonstrated that in the cells treated with *S. typhimurium* alone, the bacteria were seen individually, whereas co-incubation of epithelial cells with FN resulted in increased intracellular *S. typhimurium* present in aggregates (results not shown).

DISCUSSION

The aim of the present study was to examine the effect of adenosine, a ubiquitous endogenous signalling molecule whose levels are up-regulated during intestinal inflammation or ischaemia, on FN secretion and its polarity of secretion. We further investigated if FN modulates the adhesion and invasion of *S. typhimurium* into intestinal epithelial cells. For the study, we used human intestinal epithelial cells, T84, which are shown to be phenotypically and biophysically similar to natural intestinal crypt epithelia, and IEC-6 cells, which are non-transformed primary rat small intestinal crypt-derived epithelial cells that have similarity with normal rat and human crypt intestinal epithelial cells. We show that adenosine induced an approx. 10–12-fold induction in FN protein and that its secretion is polarized to the apical compartment of polarized intestinal epithelial cells, irrespective of the surface stimulated. The results obtained in human intestinal cells were reinforced using rat primary intestinal epithelial IEC-6 cells,

Figure 6 Adenosine and FN promotes *S. typhimurium* adherence/invasion into epithelial cells

Filter-grown T84 cell monolayers, were either stimulated with vehicle or adenosine (100 μ M) for 24 h (A and B), gently washed and incubated for 1 h at 37 °C with *S. typhimurium* SL3201 added to the apical chamber, or were co-incubated with FN (12 μ g/ml; apical) and *S. typhimurium* for 1 h at 37 °C (C and D). The number of adherent and invasive bacteria were evaluated by counting the CFU after plating them on MacConkey agar plates as described in the Experimental section. The data are representative of three independent experiments and are expressed as the means $\pm$ S.D. for triplicate dilutions. *Significantly different compared with *S. typhimurium* alone ($P < 0.01$).

where we observed a similar apically polarized induction of FN upon adenosine stimulation.

Epithelial cells, in addition to their roles in the formation of barriers and in the transport of nutrients, are responsible for the synthesis and secretion of many important proteins which may be secreted vectorially into either the apical or the basolateral compartment [28]. For example, in intestinal epithelial cells, IL-6 has been shown to be secreted in a polarized fashion upon adenosine stimulation [21,29] or in response to stress [30] and the CXC chemokines, IL-8 and GRO α , are secreted basolaterally after bacterial invasion [31]. Two types of signals are thought to be involved in apical specific targeting of plasma membrane or secretory proteins. N-Glycans have been shown to act as an apical sorting signal for secretory proteins, such as growth hormones [32]. Growth hormones, when non-glycosylated, are secreted from both surfaces of MDCK (Madin–Darby canine kidney) cells, but are secreted preferentially from apical side when glycosylated [32,33]. Moreover, clusterin, an apically secreted glycoprotein in MDCK cells, is secreted randomly from both sides upon tunicamycin treatment [33]. A second signal, a GPI (glycosylphosphatidylinositol) anchor, has been shown to be a strong apical targeting signal in polarized epithelial cells [34,35]. Most of the carbohydrate moieties of FN are complex N-glycans, which may explain its apical targeting upon adenosine stimulation. Another

explanation for the polarity of secretion could be polymorphism of the FN gene, which is caused by alternative splicing of the primary transcript of a single gene. Alternative splicing at three different domains can produce up to 20 FN isoforms [4,36]. Expression of FN splice variants appears to be both developmentally regulated and tissue specific, and treatment of various cell types with cytokines may modulate the relative amounts of FN mRNA isoforms. This regulation of FN splicing leads to the production and secretion of specific fibronectin isoforms. Alternatively, cAMP induced by adenosine stimulation may direct FN secretion to the apical surface. In intestinal epithelial cells, cAMP and Cl-dependent regulation of *trans*-Golgi network acidification has been shown to modulate both sialylation of proteins and secretory vesicle budding at the *trans*-Golgi network, which mediate apical secretion of proteins such as α -1 anti-trypsin [37].

FN is a matrix protein that is typically secreted by fibroblasts, endothelial cells, macrophages and certain epithelial cells, including intestinal epithelia [38]. FN is actively secreted by proliferating and undifferentiated intestinal epithelial cells [39,40]. FN in mature intestinal crypt epithelial cells has been reported to be localized exclusively to cell–cell contacts and regulates epithelial functions such as wound healing or restitution [38,41]. FN secretion to the apical surface, to our knowledge, has not been reported before. Expression of FN *in vivo* in primary cultures or normal and transformed established cell lines is influenced by a variety of cytokines and other low-molecular-mass compounds and is subject to complex regulatory circuits. For example, the CRE (cAMP-responsive element) was shown to mediate FN gene expression in response to cAMP, serum and human papilloma virus in several cell types [42]. Among the various mechanisms that could be involved in adenosine-induced FN, it has been shown that activation of adenosine receptors affect the intracellular levels of cAMP, and we have previously shown that in intestinal epithelial cells the adenosine-mediated response occurs via the cAMP/protein kinase A pathway [25]. Indeed, blocking the adenosine receptor using alloxazine completely inhibited adenosine-induced FN. Consequently, it seems that the effects of adenosine result from an extracellular action that is presumably A_{2b}-receptor mediated, since this is the only adenosine receptor subtype that has been shown to be present in T84 cells and in human colonic epithelial cells, and are shown to be present on both surfaces of polarized epithelia [25].

Bacteria and bacteria–epithelial cell interactions play a crucial role in orchestrating inflammatory and immune responses in the gastrointestinal tract in health and in disease. Bacterial adhesion to the host cell has been considered to be an important phenomenon not only for invasion, but also for the consequent immune response. The host factor(s) contributing to the adherence of bacteria to epithelia and the mechanism of bacterial translocation is poorly understood. FN appears to be a common host cell target as numerous pathogens possess FN binding proteins [43–46]. Because several host integrins (e.g. α 5 β 1) have binding sites for FN, the ability of micro-organisms to bind soluble FN establishes a bridge between the organism and the host cell cytoskeleton, a condition necessary for the internalization of the microbe by the cell. However, FN is a matrix protein secreted basolaterally by epithelial cells and a loss of epithelial integrity is necessary for the bacteria to access FN. We report here an aberrant secretion of a matrix protein, FN, into the lumen in response to adenosine, a nucleoside up-regulated during inflammation. We show that adenosine pretreatment of epithelial monolayer promotes *S. typhimurium* adhesion and invasion into human intestinal epithelial cells, with a subsequent up-regulation of IL-8 secretion into the basolateral compartment (results not shown). Similar results were obtained when monolayers were infected with *S. typhimurium*

in the presence of FN. Interestingly, FN has been shown to be highly up-regulated in inflammatory bowel disease in humans, and bacteria, such as enterococci, co-localize with FN [47].

In summary, using physiologically relevant epithelial model systems, we have shown for the first time that FN, a classic extracellular matrix protein that is known to be secreted into the basolateral surface, is secreted into the apical compartment of epithelial cells in response to adenosine. We further established that *S. typhimurium* invasion of epithelial cells and the subsequent inflammatory response in the form of IL-8 secretion is up-regulated by adenosine or FN treatment. This study thus identifies FN as an important mediator of immune inflammatory response that could result in increased polymorphonuclear influx into the epithelial cells. Features such as these may be related *in vivo* to the accessibility of the intestinal epithelium to enteropathogenic bacteria and the modulation of the mucosal immune responses in inflammatory processes. The mechanism of activation of the epithelial or inflammatory signalling cascade by FN thus represents a potential target for therapeutic intervention. In addition, since FN has been speculated to be involved in the cascade of events leading to inflammation in general, investigation as to how adenosine modulates FN secretion would provide crucial information for understanding the possible involvement of adenosine in inflammatory responses.

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