Polarised interleukin 8 secretion by HT 29/19A cells

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Abstract

Interleukin 8 is a neutrophil chemotactic and stimulating cytokine induced by various inflammatory stimuli, including tumour necrosis factor, interleukin 1, and endotoxin. The ability of HT 29/19A enterocytes to synthesise interleukin 8 was studied. The results show that interleukin 1 is an important stimulus for interleukin 8 synthesis and secretion by HT 29/19A cells, being more potent than tumour necrosis factor. The tumour necrosis factor and interleukin 1 induced interleukin 8 secretion by HT 29/19A cells was seen to be polarised according to the direction of stimulation. These results support the concept that mucosal cells (enterocytes) may play an important part in initiating mucosal inflammation. Furthermore, it is proposed that HT 29/19A cells constitute a tool to study stimulus directed polarised cytokine secretion.

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Interleukin 8 (IL 8) is a low molecular weight cytokine with neutrophil and T lymphocyte chemotactic and stimulating properties,^{1–3} that is synthesised and released by mononuclear cells,^{4–6} fibroblasts,⁷ keratinocytes,⁷ endothelial cells,^{9–10} hepatocytes,¹¹ retinal pigment epithelial cells¹² after stimulation by interleukin 1 (IL 1), tumour necrosis factor or endotoxin. Mitogens, lectins, and tumour promoters – that is, phorbol myristic acid, can also induce IL 8 synthesis.^{13–14} High serum concentrations of IL 8 are seen in endotoxaemia and sepsis,^{15–16} and local IL 8 production is increased in several inflammatory diseases.^{17–18} It is probable that in these conditions IL 8, in conjunction with other cytokines such as IL 1 and tumour necrosis factor, attracts and subsequently activates neutrophils to release proteases and oxygen radicals, which may lead to tissue damage. Recently, we found high concentrations of IL 8 in large bowel biopsy specimens of patients with ulcerative colitis (Hommes DW, Van Deventer SJH, unpublished data) and identified mucosal cells (enterocytes) as one of the cellular sources (Radema and van Deventer, unpublished data). Raised cytokine concentrations have been shown in the gut lumen as well as in the mucosa of patients with ulcerative colitis. One study reported high tumour necrosis factor values in stools of patients suffering from inflammatory bowel diseases during the active phase of the disease.¹⁹ Other investigations reported high concentrations of IL 1 in mucosal biopsy specimens obtained from patients with inflammatory bowel disease.^{20–21} In this context it became important to identify the stimuli that cause IL 8 secretion by mucosal cells. We therefore investigated the ability of enterocytes to respond to

cytokines by secreting IL 8 in vitro. In addition, pharmacological agents (calcium ionophores, phorbol myristic acid) were used to explore the cellular mechanisms of this secretory response. The subclone 19A²² of the human colon carcinoma cell line HT 29 was selected as model system because it forms monolayers of highly polarised cells that can respond to neuroendocrine secretagogues by secreting chloride, this functional differentiation being reminiscent of chloride secretory cells in the normal intestinal crypt.²³ In other words, this clonal cell line typifies a phenotypically and functionally differentiated epithelial intestinal cell. Another advantage for using this cell clone is that it differentiates into highly differentiated cells when cultured in standard culture medium (glucose containing medium).

Methods

CELL CULTURE

HT 29/19A cells were grown in 25 cm² cell culture flasks (Costar Cambridge, USA) in Dulbecco's Modified Eagle's medium containing 4.5 g/l glucose (Life Technologies Ltd, Paisley Scotland). This medium was enriched with non-essential amino acids (1%) (Life Technologies Ltd, Paisley Scotland), fetal bovine serum (10%), and ciproxin (0.1%).

STIMULATION

Separate experiments were performed with cells grown in wells (Falcon, nr 3047, multiwell, 24 well, Becton & Dickinson, New Jersey) and on microporous filters (Falcon nr 353095, cyclopore membrane with 0.45 µm, pore size, diameter 9 mm, Becton & Dickinson, New Jersey) placed in wells. In all experiments, the cells were stimulated after complete confluency was reached. Monolayer confluency was tested on several occasions both morphologically after Giemsa staining and by measuring the electrical resistance over the monolayer (mean resistance (SEM) was 276 (16) Ω × cm², n=19).²⁴ On each occasion, the electrical resistance was measured for three hours. Permeability studies performed in Ussing chambers showed that the agents used (including tumour necrosis factor and IL 1) did not affect the permeability of horseradish peroxidase, lactulose, and mannitol. Recombinant human tumour necrosis factor was obtained from Cetus Corporation (Emeryville, CA) and IL 1 from Medgenix (Fleurus, Belgium). Phorbol myristic acid and both calcium ionophores, ionomycin, and A23187 were obtained from Sigma Chemical Company Ltd (St Louis, USA). Several experiments were performed in this study. Firstly, cells grown in 24 well plate

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wells were stimulated with a combination of phorbol myristic acid (100 ng/ml) and a calcium ionophore (ionomycin, 50 µg/ml or A23187, 200 nM) during 2, 4, 6, and 24 hours, at which time points the amount of IL 8 secreted was measured. In similar experiments tumour necrosis factor (100 ng/ml) and IL 1 (140 pg/ml) were used as the stimulus. Also, HT 29/19A cells were stimulated by graded concentrations of IL 1 (range: 1.1 pg/ml to 140 pg/ml) and tumour necrosis factor (range: 1.1 pg/ml to 140 ng/ml). The data presented represent mean values obtained in three experiments. In a second series of experiments, confluent HT 29/19A cell monolayers grown on microporous filters were stimulated apically or basally with tumour necrosis factor (100 ng/ml), IL 1 (140 pg/ml) or the phorbol myristic acid/A23187 combination. In this experiment the stimuli were added either above (upper compartment) or below (lower compartment) the cells, and the incubation time ranged from 24 to 48 hours. These experiments were performed in duplicate or triplicate and were repeated five to eight times.

IL 8 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

IL 8 was measured with a previously published ELISA.²⁵ In brief, the ELISA wells were coated with anti-IL 8 monoclonal antibody (5 µg/ml). After removal of unbound antibody and blocking of the remaining non-specific binding sites, cell culture supernatants were added to the coated microtitre plate. After incubation, the plate was washed and a detecting antiserum enzyme conjugate of goat polyclonal anti-IL 8 antiserum was added to the wells. After further incubation and subsequent washings, the bound goat antiserum was detected with p-NPP (1 mg/ml in diethanolamine buffer). The optical density values of the samples were read at 450 nm on an automated ELISA plate reader. The detection limit of this assay in our hands was 15 pg/ml. The measured IL 8 values were corrected for the volume of the supernatant and then expressed as IL 8/ng/10⁶ cells. One well contained about 14×10⁵ cells, one filter contained about 5×10⁶ cells.

STATISTICAL ANALYSIS

Differences between means were calculated with the Mann-Whitney U test, using the SPSS package (Chicago, Illinois). All reported significance levels represent two tailed p values.

Results

IL 1 AND TUMOUR NECROSIS FACTOR STIMULATE IL 8 SECRETION

After 24 hours of stimulation with tumour necrosis factor and IL 1, HT 29/19A cells secreted IL 8, whereas non-stimulated cells produced virtually no IL 8. A time course experiment with tumour necrosis factor and IL 1 resulted in a time dependent increase of IL 8 during the first four hours after addition of the stimulus (Fig 1). IL 1 and tumour necrosis factor

Figure 1: Time dependent IL 8 secretion. Secretion of IL 8 in time after stimulation with tumour necrosis factor (TNF) and IL 1.

induced IL 8 synthesis in a dose dependent way, IL 1 being a more potent stimulus than tumour necrosis factor (Fig 2). The optimal dose for IL 1 was 4.4 pg/ml, for tumour necrosis factor 1.1 ng/ml. The IL 8 concentrations measured at these optimal stimulation doses were 1.13 (0.03) IL 8/ng/ml (for IL 1) and 1.53 (0.23) IL 8/ng/ml (for tumour necrosis factor).

POLARISED IL 8 SECRETION

Stimulation of HT 29/19A cell monolayers with tumour necrosis factor (100 ng/ml) and IL 1 (140 pg/ml) at both the apical and basolateral side (Fig 3) induced a polarised IL 8 secretion that was higher at the side where the stimulus had been added (Fig 4). Hence, addition of the stimulus (either tumour necrosis factor or IL 1) to the upper compartment caused secretion of IL 8 that was significantly higher to the apical direction. Conversely, stimulation from the lower compartment resulted in a preferential secretion of IL 8 to the basolateral direction. Tumour necrosis factor added to the upper compartment caused IL 8 secretion that was 5.49 (2.69) IL 8/ng/10⁶ cells to the upper compartment, and 1.41 (0.88) IL 8/ng/10⁶ cells to the lower compartment ($p=0.01$). Tumour necrosis factor applied in the lower compartment caused IL 8 secretion that amounted 7.96 (1.72) IL 8/ng/10⁶ cells to the lower compartment and 3.58 (1.45) IL 8/ng/10⁶ cells to the upper compartment ($p<0.01$). The amount of IL 8 secreted after IL 1 application to the upper compartment was 1.69 (0.72) IL 8/ng/10⁶ cells to the upper compartment and 2.39 (0.1) IL 8/ng/10⁶ cells to the lower compartment ($p=0.25$). IL 1 applied to the lower compartment, however, induced a much larger amount of IL 8 to be secreted to the lower compartment (8.42 (1.56) IL 8/ng/10⁶ cells) than to the upper compartment (1.78 (0.66) IL 8/ng/10⁶ cells) ($p<0.01$). At the concentrations of 140 ng/ml (tumour necrosis factor) and 100 pg/ml (IL 1), tumour necrosis factor was a more powerful inducer of IL 8 secretion than IL 1 when applied in the upper compartment, 5.49 (2.69) v 2.39 (0.1) IL 8/ng/10⁶ cells in the upper compartment ($p<0.01$). When applied below, no significant differences in IL 8 secretion to the lower compartment were seen between tumour necrosis factor (7.96 (1.72) IL 8/ng/10⁶ cells) and IL 1 (8.42 (1.56) IL 8/ng/10⁶ cells). The polarised stimulus directed pattern of IL 8 secretion by

Figure 2: Dose range stimulations. Dose dependent IL 8 secretion by HT 29/19A cells. Stimulation was performed with tumour necrosis factor (TNF) and IL 1 in the concentration ranges of 1.1 pg/ml to 140 ng/ml and 1.1 pg/ml to 140 pg/ml respectively. The cells were incubated for 24 hours in the presence of either tumour necrosis factor or IL 1.

HT 29/19A cells was seen after stimulation with either suboptimal, optimal or supraoptimal concentrations of tumour necrosis factor and IL 1. To discover if IL 1 or tumour necrosis factor caused a change in the integrity of the enterocyte

Figure 3: The application system used at the filter experiments. Stimuli were applied either in the upper compartment or in the lower compartment.

monolayer, the electrical resistance was measured. The results of these experiments suggest that the cytokines used do not change junctional permeability or cell viability (mean resistance (SEM) was 276 (16) $\Omega \times \text{cm}^2$) and did not change.

STIMULATION OF IL 8 SECRETION WITH PHORBOL MYRISTIC ACID/CALCIUM IONOPORE
After stimulation with phorbol myristic acid/ionomycin or phorbol myristic acid/A23187 combinations, HT 29/19A cells secreted large amounts of IL 8. After four hours of stimulation with phorbol myristic acid/ionomycin and phorbol myristic acid/A23187 the IL 8 concentration in the supernatant increased to 3.01 (0.51) ng/ml and 1.69 (0.05) ng/ml respectively. After six hours the IL 8 concentrations further increased to 5.72 (0.20) ng/ml and 5.9 (0.16) ng/ml, and after 24 hours to 5.86 (0.03) ng/ml and 9.64 (2.71) ng/ml (a 13 to 21-fold increase). Surprisingly, in experiments with cells grown on microporous filters the combination of phorbol myristic acid/A23187 induced only IL 8 secretion when applied in the upper compartment. No IL 8 secretion was induced when phorbol myristic acid/A23187 was added in the lower compartment (Fig 4). To discover if phorbol myristic acid/A23187 applied to the lower compartment was adsorbed by the microporous filter, we cultured HT 29/19A cells in wells and stimulated the cells for 24 hours with phorbol myristic acid/A23187, which was added in a microporous filter insert that previously had been coated with HT 29/19A matrix proteins. In this system phorbol myristic acid/A23187 induced an IL 8 secretion from the HT 29/19A cells, showing that phorbol myristic acid/A23187 was not absorbed to the microporous filter. The combined action of phorbol myristic acid/calcium ionophore did not modify the electrical resistance of the monolayers.

Discussion
Our results show that IL 8 is secreted by HT 29/19A cells after stimulation with tumour necrosis factor and IL 1. These data complement and extend previous findings showing that primary cultures of human airway epithelial cells²⁶ as well as human bronchial epithelial cell lines^{27 28} can secrete IL 8 upon stimulation with various stimuli. After stimulation with tumour necrosis factor and IL 1, the amount of IL 8 released progressively increased during the first four hours, suggesting that IL 8 was continuously produced and not merely released from internal storage. This finding is in line with other results showing that human bronchial epithelial cell lines express IL 8 in response to the inflammatory mediator tumour necrosis factor primarily by increasing the rate of transcription of the gene.²⁷ The IL 8 concentration in both experiments decreased slightly, possibly because of degradation of IL 8. The decrease seen in the IL 8 concentration may have also resulted from a cytotoxic effect, because IL 1 in concentrations higher than 140 pg/ml was toxic for HT 29/19A cells. Appreciable IL 8 induction was seen when

Figure 4: Stimulation in upper or lower compartment: polarised IL 8 secretion. Polarised stimulus directed secretion of IL 8 by HT 29/19A cells after stimulation with IL 1, tumour necrosis factor, and phorbol myristic acid/A23187 in the upper or lower compartment. Open bars represent the control wells, the striped bars represent the tumour necrosis factor stimulated wells, the hatched bars represent the IL 1 stimulated wells, and the solid bars represent stimulation with phorbol myristic acid/A23187. Phorbol myristic acid/A23187 applied in the lower compartment was restricted by the filter.

tumour necrosis factor and IL 1 were added in concentrations that can be found in the mucosa of patients with ulcerative colitis.^{20, 21} It remains unknown whether these cytokines trigger the same intracellular signalling pathways. It was recently shown, however, that viral infection can stimulate IL 8 secretion from bronchial epithelial cells in an IL 1 independent manner.²⁶ This finding supports the concept that IL 8 secretion may be triggered by several pathways.

Surprisingly, after addition of the stimuli (tumour necrosis factor, IL 1) to the upper (apical) or lower (basolateral) compartments, HT 29/19A cells could 'sense' the direction from which they were stimulated and secreted IL 8 predominantly into that direction. The mechanisms that cause such polarised IL 8 secretion are incompletely understood. In the process of transport of membrane bound or secretory proteins, reorganisation of the cytoskeleton is considered to play an important part. It is well known that cytokines, such as tumour necrosis factor, may cause a rapid change in the cytoskeletal organisation in various cells.²⁹ Cytoskeletal reorganisation that results from intercellular contact of immune cells, such as T cell-B cell contact, may direct cytokines intracellularly specifically to the area of the cell-cell contact.³⁰ Our preliminary results, however, seem to refute an important role of actin filaments in the sorting of IL 8, as cytochalasin B, a drug that interferes with the actin filaments did not perturbate its sorting (data not shown). Another hypothesis is that IL 1 or tumour necrosis factor may cause changes in the apical to basolateral area ratio upon binding to their specific receptors. In fact the possibility exists that such changes may occur because it has recently been shown that an exogenous stimulus – that is, a different growth support – can induce a modification of the apical to basolateral surface area ratio of cultured MDCK cells.³¹ Consequently the secretory rate of IL 8 would be increased at the side showing the maximal surface area.

The combined action of phorbol myristic acid/calcium ionophore resulted in IL 8 secretion by the HT 29/19A cells. Phorbol myristic acid/calcium ionophore as a stimulus showed a time dependent IL 8 secretion similar to tumour necrosis factor. The phorbol myristic acid/calcium ionophore combination, however, was only effective in stimulating IL 8 release when applied to the upper compartment. Experiments performed to check if phorbol myristic acid/A23187 applied in the lower compartment was restricted by the microporous filter, showed that the lack of IL 8 secretion could not be explained by adsorption of phorbol myristic acid/A23187 to the microporous filters. After 24 hours of apical stimulation phorbol myristic acid/A23187 presented within a microporous insert could induce IL 8 secretion comparable with phorbol myristic acid/A23187 applied on the cells. One possible explanation for this finding is that binding of phorbol myristic acid to phospholipids in the apical membrane of HT 29/19A cells, in particular phosphatidylserine,³² is necessary to induce signal transduction and initiate gene transcription. Viral transfection studies in MDCK epithelial cells are in accordance with this hypothesis because viruses that budded from the apical plasma membrane were enriched in phosphatidylserine, whereas viruses budding from the basolateral surface were not.³³ It has recently been reported that apical application of phorbol myristic acid, but not basolateral application, induced Cl⁻ secretion by T84 mucosal cells, and this result led the authors to conclude that phorbol myristic acid induced an apical subcellular pool of protein kinase C.³⁴

Presumably, IL 8 plays an important part in inflammatory bowel disease, in particular in ulcerative colitis, which is histologically characterised by large amounts of infiltrating neutrophils. Increased IL 8 mRNA concentrations were detected in mucosal biopsy specimens of patients suffering from inflammatory bowel disease.³⁵ The IL 8 that is released basolaterally by enterocytes may participate in the recruit-

ment and subsequent activation of neutrophils.³⁶ At present we have no explanation for the biological importance of apical secretion of IL 8 by enterocytes. Interestingly, it has been recently reported that IL 1 can be a growth factor for *Escherichia coli*,³⁷ and bacteria can produce an IL 1 like activity themselves.³⁷ IL 1 has been detected in mucosal cells in vivo^{20,21} and in non-differentiated mucosal cells in experimental colitis.³⁸ Enterocytes and bacteria may therefore interact by means of cytokine secretion. Although the mucosal cytokine networks have been incompletely characterised, the ability of mucosal cells to synthesise IL 1 and IL 8 is in accordance with the hypothesis that enterocytes, in addition to endothelial cells, peripheral blood monocytes infiltrated in the mucosa, and resident lamina propria mononuclear cells play an important part in the mucosal inflammatory cascade. In conclusion, HT 29/19A cells can produce large amounts of IL 8 after stimulation by IL 1 and tumour necrosis factor, at concentrations that may occur in inflammatory bowel disease. IL 8 secretion by HT 29/19A cells is polarised according to the direction of the stimulus. Therefore, HT 29/19A cells may be used to investigate the mechanisms of polarised cytokine release in more detail.

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