Interleukin 8: cells of origin in inflammatory bowel disease

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Abstract

Neutrophils are important cellular mediators in inflammatory bowel disease (IBD). Interleukin (IL)8, a powerful neutrophil chemoattractant, is found in increased quantities in inflamed mucosa, but the cells of origin are uncertain. IL8 gene expression was studied by in situ hybridisation in uninfamed intestinal tissue resected for colon carcinoma (n=7) and in inflamed colonic tissue resected for IBD (n=11). Immunohistochemistry was used to assess the phenotype of IL8 expressing macrophages and the production of IL8 protein. Macrophages isolated from intestinal resections and lipopolysaccharide stimulated peripheral blood monocytes treated with 5-aminosalicylic acid, hydrocortisone, and cyclosporin A were examined for IL8 mRNA by northern blotting and IL8 secretion by enzyme linked immunosorbent assay (ELISA). In all cases IL8 mRNA was detected by in situ hybridisation in macrophages and neutrophils adjacent to ulceration in inflamed bowel, but not detected in uninfamed mucosa from carcinoma resections. Recently recruited CD14 positive macrophages were responsible for some of this IL8 expression. IL8 protein was present in the same distribution as mRNA. Epithelial cells in normal and inflamed tissue showed neither mRNA nor protein. IL8 mRNA was expressed significantly more commonly by macrophages from IBD affected than from normal mucosa, and IL8 secretion by IBD but not normal colon macrophages was augmented significantly by lipopolysaccharide treatment. IL8 expression and production by lipopolysaccharide treated blood monocytes was inhibited by the therapeutic agents tested. These results show that neutrophils and recently recruited macrophages are responsible for production of IL8 in IBD, suggesting a mechanism for a continuing cycle of neutrophil attraction. Agents used therapeutically in these diseases may be effective in part by disrupting this cycle.

(Keywords: interleukin 8, macrophage, polymorphonuclear leucocyte, inflammatory bowel disease, in situ hybridisation, immunohistochemistry.)

Inflammatory bowel diseases (IBD) are characterised by the accumulation of inflammatory cells, including neutrophils, monocytes, lymphocytes, and plasma cells, in the lamina propria of affected intestine. While many of these cells have potential effector functions in mediating continuing tissue injury, neutrophils play a major part in acute mucosal inflammation because of the enormous numbers present in inflamed intestine and because of their potent ability to produce toxic mediators such as reactive oxygen and nitrogen intermediates as well as cytokines.

Neutrophil turnover in active IBD has been shown by radioisotope labelled leucocyte scans to be greatly increased. Some of the signals responsible for the attraction and activation of neutrophils, including leukotriene B4, platelet activating factor, granulocyte-macrophage colony stimulating factor (GM-CSF), and interleukin (IL)8 have been shown to be increased in the serum and inflamed mucosa of patients suffering from active IBD. The relative contributions of the different neutrophil chemoattractants however, is not clear.

IL8, a member of the α chemokine family of chemoattractant cytokines, is a potent chemoattractant and activator of neutrophils. It is produced by a wide variety of cell types, including macrophages, neutrophils, T cells, endothelial cells, keratinocytes, fibroblasts, hepatocytes, and chondrocytes in vitro (reviewed in ref 11), and is found in various disease states, including psoriasis, rheumatoid arthritis, occlusive vascular disease, and septic shock. In addition, there is good evidence that some transformed intestinal epithelial cell lines can be stimulated to produce IL8 as can primary cultures of intestinal epithelial cells but whether mucosal epithelial cells produce IL8 in the IBD intestinal lesion is not clear.

Despite the findings of increased IL8 in IBD and the large number of cell types capable of producing this cytokine, the cells responsible for its expression and secretion in the inflamed intestinal environment are yet to be elucidated. We report here that recently recruited mucosal macrophages and neutrophils but not epithelial cells are prominent producers of IL8 in actively inflamed IBD tissue as shown in situ, suggesting a positive feedback loop of neutrophil attraction in these diseases. Isolated intestinal macrophages also express and secrete IL8, and agents used in the treatment of IBD are capable of inhibiting IL8 production, suggesting that at least part of their efficacy may result from inhibition of neutrophil recruitment.
Methods

Patients
Resected intestinal tissue was obtained from 16 patients suffering from Crohn's disease and eight suffering from ulcerative colitis. The mean age of IBD patients was 27 years (range 17-49). All were being treated with corticosteroids at the time of surgery; five of eight ulcerative colitis patients were being treated with sulphasalazine or 5-aminosalicylic acid and two of the Crohn's disease patients were receiving azathioprine. Histologically uninflamed intestinal tissue was obtained more than five centimetres from carcinoma in 19 patients undergoing surgery for colonic cancer. The mean age of these patients was 63 years (range 52-75).

IL8 in situ hybridisation
Sense and antisense riboprobes for IL8 were produced from an IL8 cDNA (450 bp EcoRl fragment in pUC 19 vector – generous gift from Dr Mark Smyth, Austin Hospital, Melbourne, Australia) by restriction enzyme digestion and subcloning into the EcoRl site of the pGEM-4Z vector (Promega, Madison, WI, USA). The plasmid was linearised using EaeI or SmaI and labelled with 35S-UTP by transcription using T7 or SP6 RNA polymerases (Promega), to generate antisense and sense riboprobes respectively. These then were cut into approximately 100 bp fragments by alkali line hydrolysis. In situ hybridisation of intestinal tissue was performed as described previously. Briefly, formalin fixed, paraffin wax embedded tissue from IBD (six Crohn’s disease and five ulcerative colitis) and un-inflamed, cancer bearing colons (n=7) was cut into 3 μm sections, dewaxed, and treated for 20 minutes in 0.2 M HCl. Sections then were permeabilised using proteinase K 50 μg/ml (Sigma, St Louis, MO, USA), fixed in 4% paraformaldehyde, and acetylated using acetic anhydride 0-2% in 100 mM triethanolamine, before dehydrafication in alcohol and overnight hybridisation at 47°C with sense or antisense IL8 riboprobes in 50% formamide, 1x Denhard's solution, 0.3 M NaCl, 5x10^-4 M EDTA, 0.01 M TRIS, 5x10^-3 M Na2HPO4, 5x10^-13 M NaH2PO4, 10% dextran sulphate, transfer RNA 1 μg/ml (Sigma, St Louis, MO) and 10 mM dithiothreitol (DTT; Kodak, New haven, CT, USA). Subsequently sections were washed in hybridisation solution at 50°C then NTE (NaCl 0.5 M, TRIS pH 7.2 10 mM, EDTA 1 mM) and 10 mM DTT, treated with RNase A 20 μg/ml (Pharmacia LKB, Piscataway, NJ, USA) in NTE at 37°C and washed in NTE and in 0.1xSSC/1 mM DTT, then coated with photographic emulsion (K5 gel emulsion, Ilford, Cheshire, UK) and exposed for up to two weeks at 4°C. The film was developed and the sections stained with haematoxylin and eosin.

Immunohistochemistry for CD14 and IL8
Serial sections from seven of the formalin fixed tissues used for in situ hybridisation (three Crohn’s disease, three ulcerative colitis, and three uninflamed from carcinoma resection) were examined simultaneously for expression of the monocyte cell surface marker CD14, which identifies mucosal macrophages recently recruited from the circulation to the intestine, as well as weakly staining neutrophils and B lymphocytes. Sections were dewaxed (xylene, rehydrated, and microwave treated at 600 W for 10 minutes in 6 M urea to re-expose cross linked antigen). Endogenous peroxidase activity was quenched by incubating sections with 0.3% hydrogen peroxide in methanol. Subsequent incubations were performed at room temperature in a moist chamber. Sections were treated with 4-5% normal horse serum and then incubated overnight with murine monoclonal antibodies directed against monocytes and macrophages (anti-CD68, Dakopatts, Denmark) and against CD14 (CMRF31: binds to CD14 transflectants, blocks lipopolysaccharide binding – Calder et al, maruos gift; generous gift from Dr Derek Hart, Department of Haematology, Christchurch Hospital, Christchurch, New Zealand), and washed three times. No antibody and irrelevant matched isotype antibodies were used as negative controls. The slides were incubated with a 1:200 dilution of sheep antimouse biotinylated antibody (Amersham International, UK) in phosphate buffered saline (PBS), washed three times in PBS, incubated with avidin/biotinylated horseradish peroxidase complex (ABC, Vector Laboratories, CA, USA), and washed with PBS. Slides then were stained with 3,3'-diaminobenzidine (0.5 mg/ml, Stansens, Australia), imidazole (10 mM), and hydrogen peroxide (0.3%) for five minutes, washed in tap water for five minutes, counterstained with Harris' haematoxylin, and mounted using Histoclad (Clay Adams, Parsippany, NJ, USA). Despite repeated attempts to define conditions that allowed immunohistochemistry for CD14 to be performed on the same tissue sections as in situ hybridisation, morphological results suitable for analysis could not be obtained. Thus serial sections were examined to determine apparent coexpression of IL8 mRNA and CD14 by monocytes/macrophages.

For IL8 immunohistochemistry, colonic tissue from four Crohn’s disease, two ulcerative colitis, and four colon carcinoma resections was fixed immediately in 2% periodate-lysine paraformaldehyde (PLP) for four hours before overnight washing in 7% sucrose phosphate buffer. Fixed tissue was embedded in OCT compound (Tissue-Tek, Miles Inc, IN, USA) and stored at −20°C. Sections of 5 μm were cut, dried, washed to remove OCT, and examined for expression of IL8 using the histochemical methods described above. In the above method, a polyclonal rabbit antibody against IL8 (generous gift from Dr Steven Kunkel, University of Michigan Medical School, Ann Arbor, MN) and biotinylated sheep-antirabbit IgG (Serotec, Oxford, UK) at a dilution of 1:400 in PBS. Negative controls used included no primary antibody,
pre-immune rabbit serum, irrelevant rabbit polyclonal antiserum, and anti-IL8 pre-adsorbed with recombinant human IL8, as described.  

Subsequently IL8 staining was performed on formalin fixed tissue (six Crohn's disease, five ulcerative colitis, and seven colon cancer resections, as for in situ hybridisation) treated with 6 M urea as described above.

**Intestinal mucosal disaggregation and macrophage and T cell enrichment**

Intestinal mucosa was disaggregated enzymatically as previously described. Briefly, mucosa was stripped from submucosa within 30 minutes of resection, and washed several times in calcium-magnesium free Hanks's buffered salt solution with 0.75 mM EDTA, then without EDTA. The tissue was minced finely, and incubated overnight in RPMI 1640 (Flow Labs, Australia) with 10% fetal calf serum, 2 U/ml collagenase (CLS-PA type, Worthington Biochemical Corp, Freehold, NJ, USA), and 5 U/ml DNase II (Calbiochem, San Diego, CA, USA). The digest was filtered, then centrifuged through Ficoll-Paque (Pharmacia Fine Chemicals AB, Uppsala, Sweden). The resultant mononuclear cell preparation was >85% viable by 0.1% trypsin blue exclusion.

Macrophages were enriched by fibronectin or γ globulin adherence as described previously. The resulting adherent cells were 75–80% macrophages as determined by staining with the anti-macrophage/macrophage marker anti-CD68 with viability >80%. Virtually all contaminating cells were T lymphocytes that stained with anti-CD3 (OKT3, Ortho-mune, NJ, USA).

Intestinal T cells were enriched from the mononuclear cell population by 2-aminoethylisothiourea bromide (AET) sheep red blood cell rosetting as previously described. The resulting population of >85% T cells, as determined by staining with anti-CD3, showed viability values >90%.

All materials used were free of contamination by lipopolysaccharide as determined by limulus amoebocyte assay.

**RNA extraction and northern blotting**

Total cellular RNA from macrophages and T cells were extracted by the method of Chomczynski, quantified by determining optical density at 260 nm, and stored at −70°C. RNA (5 μg per lane) was electrophoresed in 1% agarose-formaldehyde gels and blotted onto nitrocellulose filters. The cDNA for IL8 was labelled with α32P-dCTP by random priming and used to probe northern blots. A similarly labelled cDNA for 18S ribosomal (r)RNA was used as a control to determine equal loading of lanes. Non-specific radioactivity was removed by washing the blots in 2×SSC (0.3 M NaCl, 30 mM trisodium citrate) and 0.1% sodium dodecyl sulphate at 60°C. Labelled blots were exposed to radiography film (XAR5, Eastman Kodak, Rochester, NY, USA) at −70°C for one to four days then developed.

**IL8 secretion by isolated intestinal macrophages**

Isolated macrophages from four IBD resections (two ulcerative colitis and two Crohn's disease) and from five uninfamed colon cancer resections were enriched by adherence to human γ globulin. Adherent macrophages at 1×10^6/ml were stimulated for six hours by sonicated lipopolysaccharide (Re 595 from Salmonella minnesota) at concentrations of 0, 1, 10, and 100 ng/ml in 1 ml RPMI 1640+10% fetal calf serum. Conditioned media were harvested after six hours and stored at −70°C for subsequent IL8 measurement. IL8 enzyme linked immunosorbent assays (ELISAs) were performed in triplicate according to the manufacturer's instructions (Quantikine, R&D Systems, Minneapolis, MN, USA).

**Lipopolysaccharide stimulated peripheral blood monocytes**

Peripheral blood was collected from three healthy volunteer blood donors anduffy coats subjected to density gradient centrifugation with Ficoll-Paque to obtain mononuclear cells, and monocytes enriched by adherence to human γ globulin as described above. Monocytes comprised >85% of these cells and viability values exceeded 90%. Adherent monocytes were stimulated for six hours by sonicated Re 595 lipopolysaccharide at a concentration of 10 ng/ml after preliminary experiments showed optimum IL8 mRNA expression at this value (data not shown). Cells were treated simultaneously with hydrocortisone 200 μg/ml (Glaxo, Melbourne, Australia) or 5-aminosalicylic acid 500 μg/ml (Pharmacia, Sydney, Australia), or cyclosporin A 100 ng/ml (Sandoz, Switzerland), or all three, as previously outlined. Conditioned media were harvested after six hours and stored at −70°C for later IL8 measurement by ELISA and RNA was extracted, electrophoresed, and blotted as described above, probed with IL8 and 18S rRNA cDNA probes then exposed to radiography film at −70°C.

**Statistics**

Data are expressed as mean (SEM) and statistical analysis performed using analysis of variance with a Fisher's protected least significant difference post hoc test. Nominal data were compared by χ² analysis. Significance was considered to be present if p<0.05.

**Ethics**

The acquisition and use of human blood and tissue for this study was approved by the ACT Health Institutional Ethics Committee.

**Results**

**IL8 in situ hybridisation**

IL8 mRNA was not detected in the seven
normal colon sections examined using the antisense probe (Fig 1a). Examination of the five ulcerative colitis and six Crohn’s disease tissues with the antisense probe consistently showed abundant mRNA for IL8 in cells morphologically and phenotypically (CD68 positive – data not shown) resembling macrophages in proximity to ulcerated epithelium, as well as in many neutrophils in lamina propria, ulcer slough, and crypt abscesses. IL8 expression was found predominantly in areas below and adjacent to active ulceration, but isolated IL8 expressing cells also were apparent in inflamed mucosa displaying intact epithelium (Fig 1b) and in a perivascular distribution (Fig 1d). In situ hybridisation using the sense probe for IL8 was repeatedly negative in all tissues examined (Fig 1c and e). There was no demonstrable IL8 mRNA in epithelial cells, either adjacent to or away from areas of ulceration in IBD tissues (Fig 1b), nor was IL8 expressed in normal colon epithelial cells (Fig 1a).

In three experiments serial sections stained with the anti-CD14 antibody, CMRF31, showed that many of the IL8 mRNA expressing macrophages also seemed to express the monocyte marker CD14, suggesting their recent arrival from the circulation as previously described21 (Fig 2a and b).
failed to show IL8 production by colon epithelial cells.

Northern blots of isolated lamina propria mononuclear cells and peripheral blood monocytes
To confirm intestinal macrophages as IL8 expressing cells, macrophages were isolated from resected intestine. Macrophages from normal intestine expressed mRNA for IL8 in three of 19 cases, while those isolated from IBD tissue expressed IL8 in 20 of 24 cases ($\chi^2=19.44$, $p<0.0001$) (Fig 4a). To control for contaminating T lymphocytes in the isolated macrophage populations, T cells isolated from both normal and inflamed colon also were probed but no IL8 mRNA expression was detected (Fig 4a).

When a model of the recently recruited mucosal macrophage, the lipopolysaccharide treated monocyte, was treated with agents used in the treatment of IBD, IL8 expression was changed. Exposure to hydrocortisone resulted in reduced IL8 mRNA values, although the decrease was less noticeable than that seen for cyclosporin A. In addition, 5-amino salicylic acid reduced IL8 mRNA expression in lipopolysaccharide treated monocytes, both in the absence and in the presence of hydrocortisone (Fig 4b).

**IL8 secretion by isolated intestinal macrophages and by peripheral blood monocytes**
Macrophages isolated from uninflamed colon cancer bearing resections produced low concentrations of IL8 protein, which were not augmented by stimulation using lipopolysaccharide (Fig 5). Macrophages from IBD affected colons produced significantly greater quantities of IL8 than those from uninflamed colons ($p<0.005$), and this secretion was significantly increased by stimulation using lipopolysaccharide ($p=0.01$; Fig 5). There was no difference between ulcerative colitis and Crohn’s disease in the quantity of IL8 generated (data not shown).

To ensure that the observed changes of IL8 mRNA expression in lipopolysaccharide stimulated monocytes were reflected in IL8 protein production, peripheral blood monocytes were stimulated for six hours using lipopolysaccharide. Hydrocortisone, 5-aminosalicylic acid, hydrocortisone plus 5-aminosalicylic acid, and cyclosporin A all produced significant decreases in the quantities of IL8 secreted by lipopolysaccharide stimulated cells (Fig 6).

Discussion
This study confirms and extends earlier studies by showing the cell types involved in IL8 protein production as well as mRNA expression in the mucosa of active IBD. Recently there has been increasing evidence for an active role for neutrophils as mediators in inflammatory reactions, including the production of IL 1β, IL 6, GM-CSF, tumour necrosis factor (TNF) α, and IL 1 receptor antagonist,

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**Figure 2: CD14 positive macrophages express IL8 mRNA.**
(a) Subulceration mononuclear cell express mRNA for IL8 in ulcerative colitis (arrows); (b) serial section stained immunohistochemically using the anti-CD14 monoclonal antibody CMRF31 suggests that at least one of the IL8 expressing cells is a recently recruited monocyte/macrophage (arrow). Magnification: 800 X.
Figure 3: IL8 by immunohistochemistry. (b), (d), and (f). Normal, ulcerative colitis, and Crohn's disease colon stained with anti-IL8 pre-adsorbed with recombinant human IL8. No cells stain positively; (a) normal colon stained with anti-IL8 showing no positive cells in the lamina propria; (c) ulcerative colitis colon stained with anti-IL8, showing neutrophils in a crypt abscess with associated IL8. Epithelial cells appear to contain no IL8; (e) Crohn's disease colon stained with anti-IL8 showing strong positivity in lamina propria macrophages (CD68 positive on serial sections). Epithelial cells again appear negative. Magnification: 400×.
in addition to their well described passive, pro-inflammatory actions. We have shown that neutrophils in ulcerated, inflamed mucosa produce substantial quantities of IL8, using the complementary methods of in situ hybridisation and immunohistochemistry. The significance of this finding in IBD is that it implicates neutrophils in a potential cycle of attraction, activation, and subsequent production of IL8, permitting further attraction of neutrophils.

In situ hybridisation permits the demonstration of mRNA expression in unchanged tissue and when performed using riboprobes provides exquisite sensitivity. A further advantage is the delineation of all the cells of origin for individual cytokines in tissue sections. Indeed, in situ hybridisation and immunohistochemistry here show macrophages as important sources of IL8 in ulcerated IBD tissue.

This paper establishes that macrophage production of IL8 in IBD is associated with the expression of the marker CD14. CD14 is a surface molecule usually absent from intestinal macrophages but present on increased numbers of cells in IBD and a marker of the recent recruitment of monocytes from the circulation, in addition to being present on the surface of neutrophils and B lymphocytes. CD14 acts as a receptor for lipopolysaccharide bound to its carrier lipopolysaccharide binding protein and it allows macrophages to be activated by pg/ml concentrations of lipopolysaccharide. Its absence from normal intestinal macrophages probably results in their reduced responsiveness to low concentrations of lipopolysaccharide. More importantly, however, its presence on intestinal macrophages expressing IL8 shows that recently arrived monocytes contribute to the continuing attraction of neutrophils to IBD mucosa, and suggests a mechanism by which lipopolysaccharide in the inflamed mucosal...
environment may stimulate neutrophil recruitment.

Significantly, IL8 expression is confined largely to areas of ulceration or those closely adjacent; inflamed mucosa displaying intact epithelium shows little IL8 production. This finding may relate to the quantity of lipopolysaccharide and other bacterial products that have ready access to lamina propria cells in regions where the epithelial barrier is disrupted, resulting in the stimulation of pro-inflammatory and chemotactic cytokine production thereby attracting further inflammatory cells into the mucosal lesion. The subsequent destructive effects of newly arrived neutrophils may extend the ulceration increasing exposure of the lamina propria to lipopolysaccharide in a positive feedback loop.

We show also that the isolated intestinal macrophage mirrors the in vivo expression of IL8 in IBD. Compared with those from normal colon, significantly greater numbers of IBD mucosal macrophage preparations express IL8 mRNA. Similarly, macrophages isolated from IBD affected mucosa produce greater quantities of IL8 protein than macrophages from uninfamed colon cancer bearing mucosa and while the latter cells seem not to produce more IL8 after stimulation using lipopolysaccharide, IBD macrophages respond to lipopolysaccharide by a significant increase in IL8 secretion. This is an important finding and provides a functional correlate for our finding that IBD macrophages, but not macrophages in normal colon, strongly express CD14.

Alhough it has been shown previously that corticosteroids and cyclosporin may inhibit IL8 production we have shown also that 5-aminosalicylic acid down regulates the expression and production of IL8 in a model of the recently recruited intestinal macrophage, the lipopolysaccharide stimulated, CD14 positive blood monocyte. These data provide a further insight into the mechanism of action of the agents used in the treatment of IBD, and suggests that they work, at least in part, by reducing the attraction of a fresh population of neutrophils to inflamed mucosa.

An important finding from this study is the absence of detectable IL8 production by epithelial cells in both normal and inflamed colon. Epithelial cell IL8 production has been suggested by the finding that some, although not all, lipopolysaccharide stimulated colon cancer cell lines produce appreciable quantities of IL8. One of these studies has also suggested also that epithelial cell crypts isolated from normal colons produce IL8, but the crypt cell population may have been contaminated by subepithelial macrophages, as the marker used to exclude macrophage contamination was CD14, which is not seen on normal colonic macrophages. Indeed we also have been able to show IL8 production by isolated colonic crypts, but immunohistochemical analysis of these crypt preparations shows significant contamination by non-epithelial cells, especially macrophages (unpublished findings). Moreover the finding of IL8 secretion by isolated crypt epithelial cells may be an artefact. A recent preliminary report suggests that the process of isolating crypt epithelial cells from human intestine strongly induces IL8 transcription during the first hours of culture in vitro. Ignoring these caveats and given the sensitivity of riboprobe generated in situ hybridisation, if epithelial cells do produce IL8 in IBD mucosa it is probably in quantities insufficient to add significantly to the amount expressed by macrophages and neutrophils.

This study has shown a link between the analysis of cell populations isolated from colonic crypts and the determination of cytokine profiles in the tissue using in situ hybridisation and immunohistochemistry. Work now in progress is aimed at elucidating some of the signals responsible for the attraction of other inflammatory cells to IBD affected mucosa; combined with this study it offers the potential for future treatment directed at blocking the recruitment of leucocytes in IBD.

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Grimm, Elsbury, Pavli, Doe

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