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Synergic production of neutrophil chemotactic activity by colonic epithelial cells and eosinophils

Gordon Dent*, Sam C. Loweth, Anwar Matar Hasan, Fiona M. Leslie

Institute of Science and Technology in Medicine, Keele University, Keele, Staffordshire ST5 5BG, UK

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ABSTRACT

The presence of eosinophils in the lumen and mucosa of the intestine is characteristic of both ulcerative colitis (UC) and Crohn's disease (CD). There is evidence of eosinophil activation in the intestine during acute inflammatory episodes of these diseases; these episodes are also characterized by an influx of neutrophils, which have the potential to cause extensive tissue damage. We undertook a study to determine whether eosinophils in contact with colonic epithelial cells produce factors that may attract neutrophils in response to immunological stimulation. Neutrophil chemotactic activity (NCA) and concentrations of three neutrophil-attracting CXC chemokines – CXCL1 (Groα), CXCL5 (Ena78) and CXCL8 (IL8) – were measured in supernatants of T84 colonic epithelial cells and blood eosinophils or eosinophil-like myeloid leukaemia cells (AML14.3D10), alone or in combination. Cells were stimulated with serum-opsonized zymosan (OZ) particles. NCA ($P<0.005$) and CXCL5 levels ($P<0.05$) in the supernatants of OZ-stimulated epithelial/eosinophil co-cultures were significantly higher than in the supernatants of either cell type alone. Release of CXCL1 ($P<0.05$) and CXCL8 ($P<0.01$) from OZ-stimulated co-culture supernatants was significantly higher than from OZ-stimulated eosinophils but not higher than from OZ-stimulated epithelial cells. Eosinophils and colonic epithelial cells exhibit synergy in production of neutrophil chemoattractants in response to immunological stimulation. This may represent a mechanism for exaggerated recruitment of neutrophils to the intestine in response to acute infection in conditions that are characterized by the presence of eosinophils in the bowel.

Introduction

Eosinophils are associated with the chronic inflammation of the intestine in both ulcerative colitis (UC) and Crohn's disease (CD) (Baumgart et al., 1998; Nishitani et al., 1998; Wedemeyer and Vosskuhl, 2008), where they have been proposed to contribute to ulceration in the colonic mucosa and intestinal remodelling, respectively (Wedemeyer and Vosskuhl, 2008; Lampinen et al., 2004). Acute severe inflammation in UC may involve activation of eosinophils within the mucosa and the intestinal lumen, with secretion of eosinophil granule proteins, including major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil peroxidase (EPO), being of particular pathological significance (Wedemeyer and Vosskuhl, 2008; Raab et al., 1998; Forbes et al., 2004; Furuta et al., 2005). Moreover, these episodes are characterized by an influx of neutrophils, which have the capacity to cause extensive tissue destruction (Lebeis et al., 2008). By exhibiting heightened reactivity and stimulating generation of neutrophil chemoattractants, eosinophils have the potential to effect these severe episodes of inflammation.

In other systems, interaction between eosinophils and epithelial cells leads to enhanced release of inflammatory mediators, including chemokines and leukotrienes (Dent et al., 2000; Jawien et al., 2002; Wong et al., 2005). This interaction appears to be dependent upon direct cell–cell contact, probably involving ligation of eosinophil β-integrins by epithelial adhesion molecules, whose expression is up-regulated in inflammatory bowel diseases (IBD) (Vainer et al., 2000). The potential therefore exists for eosinophils to mediate inflammatory events characteristic of UC.

The aim of the present study was to determine whether interaction between eosinophils and colonic epithelial cells leads to enhance production of neutrophil chemoattractants, which would indicate a potential role for such interactions in acute severe episodes of inflammation in IBD. The study utilized the T84 colonic

Abbreviations: CD, Crohn's disease; fMLP, formylmethionylleucinylphenylalanine; IBD, inflammatory bowel disease; NCA, neutrophil chemotactic activity; OZ, opsonized zymosan; UC, ulcerative colitis.

* Corresponding author at: School of Medicine, Keele University, Keele, Staffordshire ST5 5BG, UK. Tel.: +44 0 1782 734652; fax: +44 0 1782 733634.
E-mail address: g.dent@keele.ac.uk (G. Dent).

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epithelial cell line and two types of eosinophilic cell: a transformed acute myeloid leukaemia cell line with eosinophil-like properties (AML14.3D10) (Vainer et al., 2000; Baumann and Paul, 1998) and freshly isolated human peripheral-blood eosinophils.

Materials and methods

The study was approved by the Staffordshire Research Ethics Committee (Ref. 06/Q2604/87).

Cell cultures

T84 human colonic epithelial cells were purchased from the European Collection of Animal Cell Cultures (ECACC; Porton Down, Wiltshire, UK) and cultured in Ham's F12/Dulbecco MEM (DMEM) 1:1 with GlutaMAX™ supplemented with 15 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% foetal calf serum. Cells were grown as adherent monolayers and passaged twice before finally growing to 90% confluence in 24-well tissue culture plates. Cells were maintained in serum-free medium for a further 24 h prior to use, by which time they were fully confluent.

AML14.3D10 eosinophil myelocytes were obtained under license from Dr Cassandra Paul, Wright State University, Dayton, OH (Baumann and Paul, 1998). Cells were cultured in RPMI 1640 with GlutaMAX™ supplemented with 1 mM sodium pyruvate, 100 μg/mL gentamicin and 10% FCS. Cells were maintained in suspension culture at a density below 1.5×10^6/mL and were serum-starved for 24 h prior to use in experiments. Eosinophils were isolated from peripheral blood of non-asthmatic, atopic volunteers as described (Dent *et al.*, 1998). AML14.3D10 cells and freshly prepared blood eosinophils were suspended in serum- and antibiotic-free HEPES-buffered Ham's F12/DMEM immediately before use in co-culture experiments.

AML14.3D10 cells or peripheral blood eosinophils (1×10^5/well) were added to 24-well tissue culture plates coated with BSA (1% for 60 min) or T84 colonic epithelial cells. Control wells contained T84 cells alone. Cells were incubated at 37 °C for 30 min prior to the addition of serum-opsonized zymosan (OZ) or an equivalent volume of medium. An opsonized zymosan concentration of 1 mg/mL was used, which has been demonstrated previously to be optimal for stimulation of respiratory burst in human eosinophils (Dent *et al.*, 1994) and chemoattractant release from bronchial epithelial cells (Lightfoot *et al.*, 2006). Cells were incubated for a further 24 h, after which the contents of the wells were aspirated and centrifuged ($1000 \times g$ for 2 min). Supernatants were decanted and stored at −80 °C until required for immuno- or bioassay.

Measurement of CXC chemokines

Interleukin 8 (IL8; CXCL8), growth-related oncogene α (Groα; CXCL1) and epithelial cell-derived neutrophil-activating peptide 78 (Ena78; CXCL5) were measured in culture supernatants by ELISA.

Measurement of neutrophil chemotactic activity

Human peripheral blood neutrophils were prepared as described previously (Dent *et al.*, 1997) and fluorescently labelled with calcein as described (Taylor *et al.*, 2001). Medium (negative control), 10 nM formylmethionylleucinylphenylalanine (fMLP, positive control) and culture supernatants (diluted 1:10) were added to 96-well microchemotaxis plates, which were introduced into reusable chemotaxis chambers (Neuro Probe MBA96; Receptor Technologies Ltd., Warwick, UK). Chemotaxis assays were conducted over 1 h, essentially as described (Mishra et al., 2005), using 3-μm pore-size polyvinylpyrrolidine-coated polycarbonate filters. Preliminary experiments showed 1:10 dilution of supernatants to give optimal results.

Materials

All cell culture media, supplements, foetal calf serum and calcein acetoxymethyl ester were purchased from Invitrogen Limited, Paisley, Lanarkshire, UK. fMLP and human group AB serum for zymosan opsonisation were obtained from Sigma Aldrich, Poole, Dorset, UK.

Chemokine ELISA kits were supplied by Sanquin Reagents, Amsterdam, The Netherlands (CXCL8 Pelikine Compact™) and R&D Systems, Abingdon, UK (CXCL1 and CXCL5 Quantikine®).

Zymosan A (Sigma) was opsonized as described previously (Dent et al., 2000), stored on ice and used within 1 h of preparation.

Data analysis and statistics

All data are reported as mean ± SEM. Statistical analyses were performed using StatsDirect (StatsDirect Ltd., Altrincham, UK). As not all data sets were normally distributed, data were log-transformed before analysis. Within each set of experiments, comparisons were made between OZ-induced chemokine/chemotactic activity release from each cell type or combination of cells (*i.e.* the quantity released in the presence of OZ minus the quantity released in medium alone). All conditions were analyzed using 1-way analysis of variance (ANOVA). Where ANOVA showed a significant difference among conditions, *post hoc* pairwise comparisons between conditions were performed using the Tukey–Kramer test for multiple comparisons (Motulsky, 2012). The alpha value for statistical significance was defined as $P < 0.05$.

Results

Colonic epithelial cells – but not eosinophils – secrete CXC chemokines

There was no significant difference in baseline release of CXCL8, CXCL1 or CXCL5 from T84 cells in the absence or presence of either AML14.3D10 cells or peripheral blood eosinophils (Figs. 1 and 2). T84 cells alone released significant quantities of CXCL8 (222 ± 64.0 pg/mL *vs* baseline 74.9 ± 21.6 pg/mL, $n = 12$) and CXCL1 (386 ± 88.0 pg/mL *vs* baseline 50.7 ± 10.2 pg/mL, $n = 10$; both $P < 0.001$ by paired *t* test), but not CXCL5 (3.89 ± 0.75 pg/mL *vs* baseline 2.24 ± 0.36 pg/mL, $n = 8$; $P = 0.128$), when stimulated with OZ. Neither AML14.3D10 cells alone nor peripheral blood eosinophils alone showed any significant release of any of the three chemokines in response to OZ.

Eosinophil-epithelial co-cultures do not generally secrete elevated quantities of CXC chemokines

OZ stimulated significant release of CXCL8 ($P < 0.001$ *vs* baseline, $n = 6$), CXCL1 ($P = 0.001$, $n = 6$) and CXCL5 ($P = 0.008$, $n = 4$) from T84 + AML14.3D10 co-cultures (Fig. 1). The OZ-induced release of each of the three chemokines from co-cultures was significantly higher than that from AML14.3D10 cells alone, but only CXCL5 showed significantly higher release from co-cultures than from T84 cells alone (Fig. 1).

OZ stimulated significant release of CXCL8 ($P = 0.002$ *vs* baseline, $n = 6$) and CXCL1 ($P = 0.003$ *vs* baseline, $n = 6$) but not CXCL5 ($P = 0.138$, $n = 4$) from T84 + blood eosinophil co-cultures (Fig. 2). The OZ-induced release of each of the three chemokines from