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Original Paper

Detection of Interleukin-8 mRNA and Protein in Human Colorectal Carcinoma Cells

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Interleukin-8 (IL-8) is a member of the chemokine family of pro-inflammatory chemotactic cytokines and is secreted by some human colorectal carcinoma cell lines. We have used *in situ* hybridisation and immunohistochemistry to determine whether IL-8 mRNA and protein, respectively, are produced by human colorectal carcinoma cells *in vivo*. IL-8 mRNA was detected within the cytoplasm of tumour cells in all nine samples tested, including that of a tumour which had metastasised to a lymph node. Non-involved colonic mucosa within the same tissue blocks showed much weaker labelling. IL-8 protein was detected in 74% (23/31) of tumour samples and was mainly localised to the tumour cell cytoplasm. In 30% of cases, staining was heterogeneous, with between 1 and 30% of cells being positive. In some tumour cells, IL-8 showed a perinuclear distribution resembling that found by *in situ* hybridisation. Some infiltrating leucocytes, endothelial cells and fibroblast-like cells within the tumour sections were also positive for IL-8 mRNA and protein. The possibilities that colorectal tumours produce IL-8 to aid invasion and/or metastasis or as a tumour growth factor are discussed. Copyright © 1996 Elsevier Science Ltd

Key words: interleukin-8, colorectal carcinoma, *in situ* hybridisation

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INTRODUCTION

THE CHEMOKINES comprise a family of low molecular weight pro-inflammatory cytokines involved in mediating leucocyte attraction to inflammatory sites. The human chemokines can be divided into three families based upon structural criteria and chromosomal location [1, 2]. Members of the 'C–C' family have a pair of cysteine residues that participate in disulphide bond formation with cysteines in other parts of the molecule, whereas in the 'C–X–C' family the corresponding residues are separated by a single amino acid. Genes for the human C–C chemokines are tightly linked on chromosome 17, while those for the C–X–C chemokines map to chromosome 4. Recently, a novel human chemokine, SCM-1, has been described that lacks one of the pairs of cysteine residues found in the other two chemokine families and whose gene maps to chromosome 1 [3].

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While C–C chemokines mainly attract monocytes and lymphocytes, most C–X–C chemokines are chemotactic for polymorphonuclear leucocytes [1, 2]. This function is largely attributable to those chemokines with an E–L–R motif immediately preceding the C–X–C sequence, and interleukin-8 (IL-8) is the most widely studied member of this subfamily. As well as being a neutrophil chemoattractant [4], IL-8 is also chemotactic for a subset of T lymphocytes [5, 6]. IL-8 is produced by monocytes, neutrophils and T cells, but can also be synthesised by a range of non-haematopoietic cell types, including fibroblasts, endothelial cells and keratinocytes [1, 2]. It is found in several forms, ranging from 69 to 99 amino acids in length, which differ in the degree of N-terminal cleavage. Endothelial cells predominantly produce the 77 amino acid form, while T cells and monocytes mainly produce the 72 amino acid form, which is the more potent chemoattractant. In addition to its chemotactic function, IL-8 can mediate the activation of neutrophils and monocytes. It can also induce angiogenesis by stimulating endothelial cell proliferation [7].

Epithelial cells, including normal colonic [8] and gastric epithelium [9], can also produce IL-8 and enhanced expression has been implicated in the pathogenesis of a range of inflammatory bowel disorders [9–11]. IL-8 production is also a feature of certain human tumour cell lines derived from colon carcinoma [8] and malignant melanoma [12]. Here, we have tested a panel of human neoplastic colon specimens for expression of the IL-8 gene by *in situ* hybridisation, and for the presence of IL-8 protein using a monoclonal antibody against IL-8. We report that a high proportion of colorectal tumours express both IL-8 mRNA and protein.

MATERIALS AND METHODS

Tissue samples

Routinely processed formalin-fixed, paraffin-embedded samples of normal colon, neoplastic colorectal and adjoining non-involved tissues, and a lymph node metastasis were obtained from the Department of Pathology, Royal Liverpool University Hospital and from the University of Liverpool Cancer Tissue Bank Research Centre.

Cell lines

The human colorectal carcinoma cell line HT29 was maintained in RPMI 1640, containing 10% heat-inactivated fetal calf serum, 2 mM glutamine and antibiotics (RPMI-CS). Cells were stimulated by incubation in RPMI-CS containing 1 μg/ml *Escherichia coli* lipopolysaccharide (Sigma, Poole, Dorset, U.K.) for 3 days at 37°C. T lymphoblasts were prepared by incubating human peripheral blood mononuclear cells in RPMI-CS containing 1 μg/ml phytohaemagglutinin (PHA) for 3 days at 37°C. Cells were pelleted, fixed with paraformaldehyde and embedded in paraffin, as above.

Probes

Human T lymphoblasts were prepared by incubating peripheral blood mononuclear cells in RPMI 1640, containing 10% heat-inactivated fetal calf serum and 1 μg/ml PHA, at 37°C and 5% CO_2 for 3 days. The cells were lysed and the RNA extracted and reverse transcribed in the presence of a poly-dT primer to produce cDNA. A 301 bp sequence corresponding to the entire coding region of IL-8 cDNA was amplified using the polymerase chain reaction (PCR) with a pair of oligonucleotide primers derived from the published cDNA sequence for IL-8 [13]. The forward primer had the sequence: 5′ ATGACTTCCAAGCTGGCCGTG 3′ corresponding to bases 102–122 of the published cDNA sequence. The reverse primer had the sequence: 5′ TTTTATGAATTCTCAGCCCTCTTC 3′, complementary to bases 380–403 of the cDNA sequence [13]. The PCR product was subcloned into the plasmid pCR™11 (Invitrogen, San Diego, California, U.S.A.), the resultant clones sequenced by the dideoxy chain termination method and the insert sequence shown to be identical to the published sequence [13]. For *in situ* hybridisation, control sense and antisense riboprobes were synthesised using template DNA from two clones containing the identical insert in the opposite orientation using Sp6 RNA polymerase. Reactions were carried out in the presence of unlabelled ribonucleotides and biotinylated UTP at a ratio of 1:1 with unlabelled UTP.

In situ *hybridisation*

Four-micrometre sections of paraffin-embedded tissues were dewaxed, rehydrated to 95% ethanol, and endogenous peroxidase was quenched with 1% H_2O_2 in methanol for 30 min. They were subsequently treated with 0.1 M HCl for 20 min at room temperature and incubated with 2 × SSC at 70°C for 30 min, followed by digestion with 12.5 μg/ml proteinase K (Sigma) at 37°C for 15 min. The sections were then acetylated with 0.1 M triethanolamine/0.25% acetic anhydride for 10 min at room temperature. Hybridisation with IL-8 antisense or control sense riboprobes was carried out at 42°C overnight in buffer containing 50% formamide, 2 × SSC and 10% dextran sulphate. Post-hybridisation stringency washes were 2 × SSC reducing to 0.1 × SSC at 42°C. Localisation of the probe was detected with mouse anti-biotin (Dako, Copenhagen, Denmark) diluted at 1:40 for 30 min and horseradish peroxidase-conjugated rabbit antimouse immunoglobulin (Ig) (Dako) at 1:50 for 45 min, followed by development with diaminobenzidine and H_2O_2.

Immunohistochemistry

An indirect immunoalkaline phosphatase-based method was used to detect IL-8 protein in sections of paraffin-embedded tissues. Five-micrometre sections were dewaxed, rehydrated and placed in a microwave in 10 mM sodium citrate buffer, pH 6.0, for 15 min to expose masked epitopes. Anti-IL-8 monoclonal antibody (1:20; R&D Systems, Abingdon, Oxon, U.K.) was applied overnight at 4°C. This neutralising antibody has previously been used successfully for ELISA and Western blotting (R&D Systems). After washing, the sections were incubated with biotinylated goat anti-mouse Ig (Zymed, South San Francisco, California, U.S.A.) for 30 min at room temperature followed by extrAvidin®-alkaline phosphatase (Sigma) at 1:1000 for 30 min. Antibody localisation was then visualised using naphthol-AS-MX-phosphate and fast red (Sigma). Negative control sections were treated in the same way, but were incubated overnight with an antihuman monocyte chemoattractant protein-1 (MCP-1) monoclonal antibody (R&D Systems), or with tris-buffered saline, pH 8.0, in place of the primary antibody. A mouse antihuman vimentin monoclonal antibody (Dako) was used as a positive control antibody. Duplicate sections of single blocks from 31 colorectal carcinoma patients and 1 adenoma patient were labelled on different occasions with anti-IL-8 antibody. Intensity of tumour cell labelling for IL-8 was scored by two independent observers as undetectable (−), weak (±) or strong (+) using arbitrary criteria.

RESULTS

In situ *hybridisation*

Sections from a total of 10 samples of malignant colorectal tissue were hybridised with antisense and control sense IL-8 riboprobes to detect specific mRNA. All 10 adenocarcinoma samples, including a lymph node metastasis, showed cytoplasmic hybridisation of the tumour cells with the IL-8 antisense probe. The intensity of staining ranged from weak to strong and in most cases all tumour cells within a specimen were positive (Figures 1 and 2). Some tumour cells showed a halo of perinuclear staining which was more intense than that found throughout the remainder of the cytoplasm (Figure 2). The hybridisation pattern was appar-

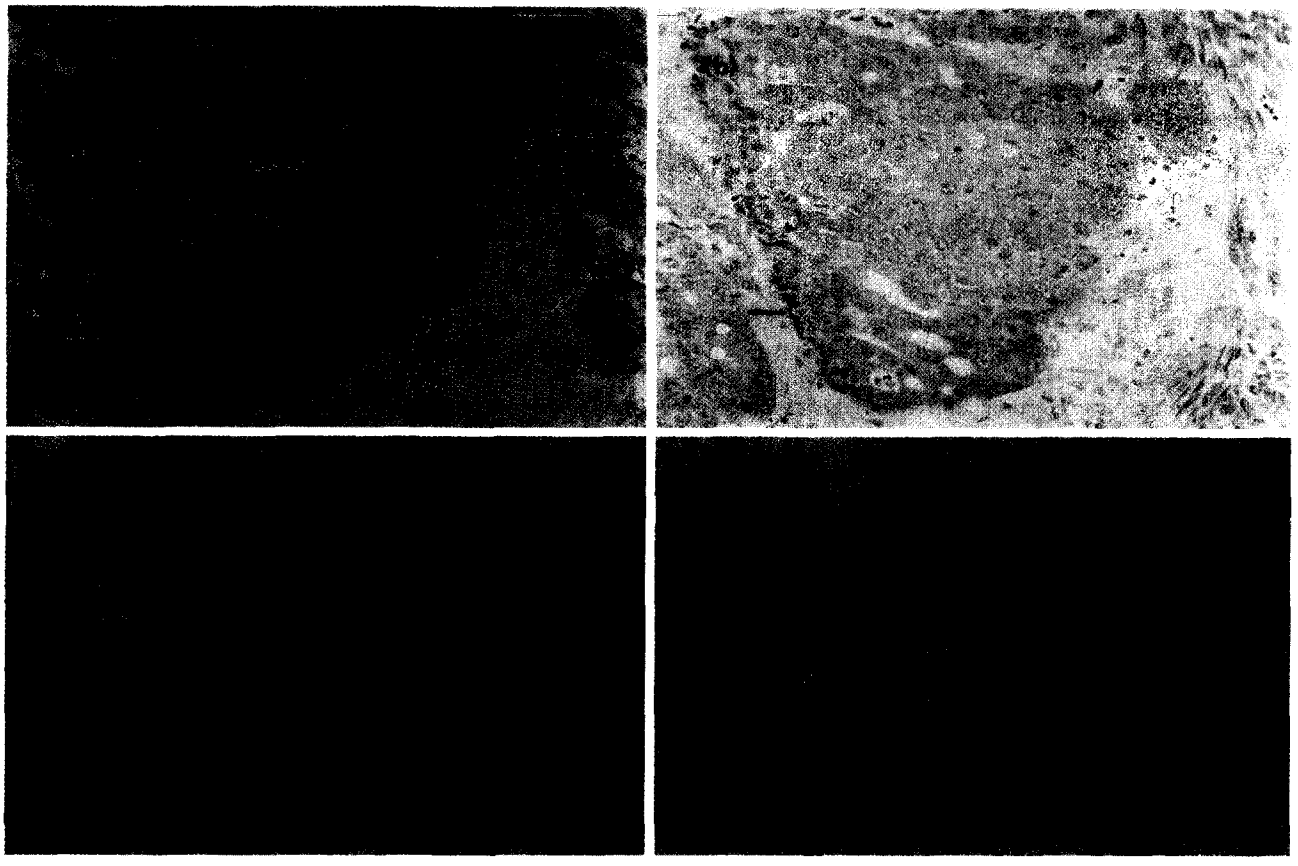

Figure 1. Strong expression of interleukin-8 (IL-8) mRNA in adenocarcinoma. *In situ* hybridisation of a moderately differentiated colon adenocarcinoma with (a) IL-8 antisense riboprobe; (b) negative control sense IL-8 riboprobe. Immunohistochemical staining of another moderately differentiated colon adenocarcinoma with (c) anti-IL-8 monoclonal antibody; (d) negative control. Bar, 10 μm.

ently unrelated to the degree of differentiation, as samples of both poorly and moderately differentiated adenocarcinomas were strongly positive (Table 1). Where adjoining non-involved tissue was present in the sections, normal colonic mucosa invariably showed much weaker staining or was completely negative. Strong, uniform cytoplasmic hybridisation was detected in tumour cells within a lymph node metastasis from a colorectal tumour patient, but no detectable hybridisation was found in the single adenoma specimen studied (Table 1). In most tumour sections, a proportion of the infiltrating leucocytes were positive, as were some endothelial cells and stromal cells of fibroblast-like morphology (data not shown). In all specimens, the control sense IL-8 probe gave very weak or negative staining.

Immunohistochemical detection of IL-8 protein

The same tumour samples previously tested for expression of IL-8 mRNA, together with an additional 21 colorectal carcinoma samples, were stained with a monoclonal antibody against human IL-8. Duplicate sections were labelled and were scored independently by two observers; agreement was exact in almost all cases. This antibody was previously tested on sections of paraformaldehyde-fixed, paraffin-embedded pellets of day 3 PHA lymphoblasts and HT29 colorectal carcinoma cells, which were found to give positive cytoplasmic staining for IL-8 (data not shown). Tumour cells from 23/31 (74%) of the colorectal carcinoma samples were positive for IL-8 protein (Table 1). Staining ranged from weak to strong and was predominantly cytoplasmic in most cases (Figure 1), but in some samples a minority of tumour cells showed perinuclear staining with occasional weak nuclear staining (Figure 2). In the majority of samples, staining of the tumour cells was uniform, but in 30% of cases it was heterogeneous with between 1 and 30% of tumour cells showing staining for IL-8 (Figure 2). With most moderately differentiated tumours, this heterogeneity of staining was found between tumour cells in the same tumour lobe, but

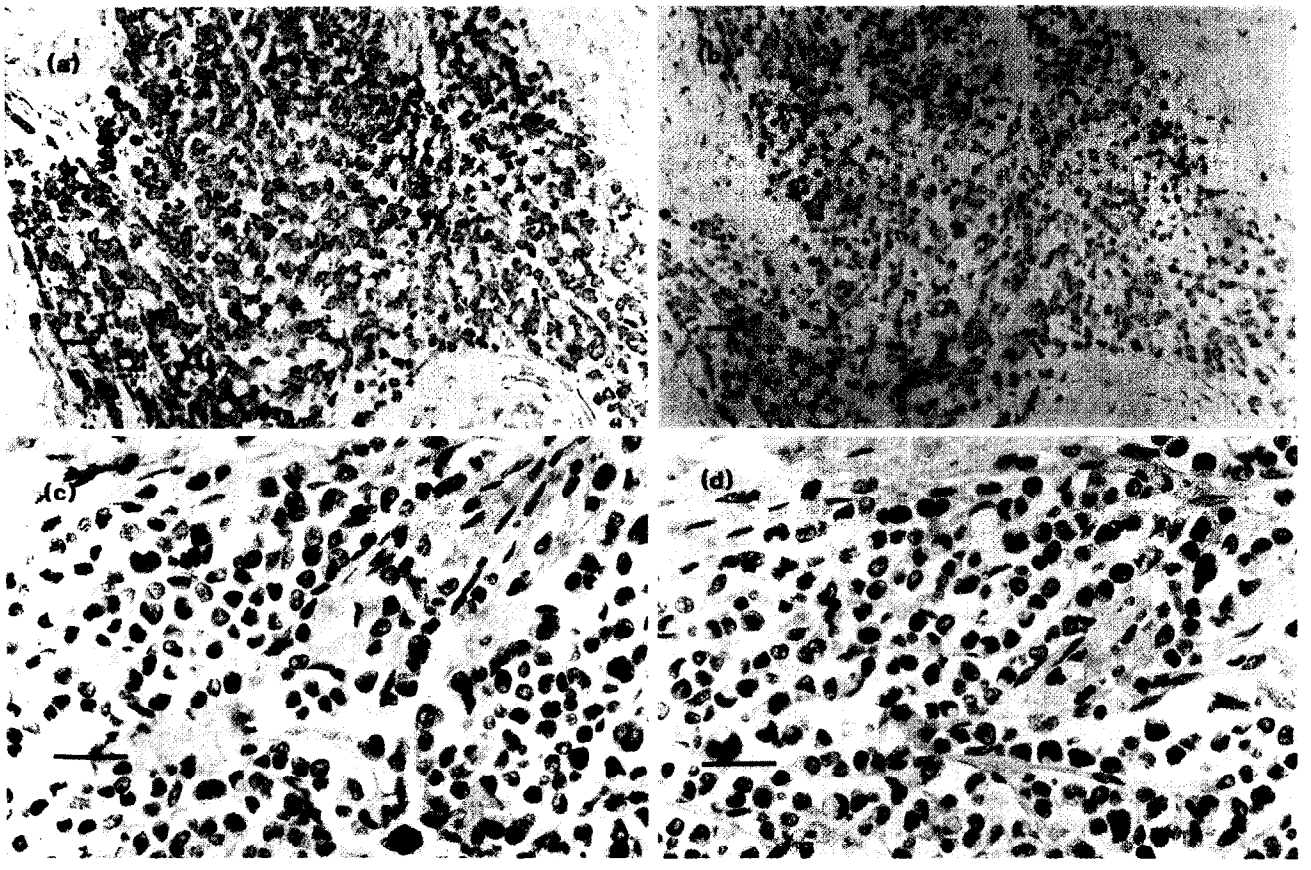

Figure 2. Strong expression of interleukin-8 (IL-8) mRNA in association with weak expression of IL-8 protein in a poorly differentiated colon adenocarcinoma. *In situ* hybridisation with (a) IL-8 antisense riboprobe; (b) negative control sense IL-8 riboprobe. Immunohistochemical staining of the same tumour with (c) anti-IL-8 monoclonal antibody; (d) negative control. Bar, 10 μm.

in one sample, neighbouring lobes were either wholly positive or negative. Two of the three mucinous carcinoma samples contained detectable IL-8. In all cases, negative control sections labelled with anti-MCP-1 antibody or with second antibody alone gave undetectable staining of tumour cells.

There was a good correlation between the intensities of staining with the antisense IL-8 probe and with the anti-IL-8 monoclonal antibody. However, several samples showed only weak staining with the antibody in the presence of strong hybridisation with the IL-8 probe (Figure 2). As in the *in situ* hybridisation studies, infiltrating leucocytes were positive in most samples; these were mostly mononuclear cells, but positive neutrophils were clearly visible in some cases. Some endothelial cells and stromal fibroblast-like cells were also positive.

Table 1. *Detection of interleukin-8 (IL-8) mRNA and protein in* 31 *colorectal adenocarcinoma specimens and one adenoma sample*

Specimen	*n*	Immunohistochemical staining for IL-8			IL-8 *in situ* hybridisation
		−	±	+	
Moderately differentiated colonic or rectal carcinoma	24*	5	6	13*	± (2/7), +(5/7)
Poorly differentiated colonic or rectal carcinoma	7	3	3	1	+ (2/2)
Adenoma	1	1	—	—	n.t.

*Including a lymph node metastasis of a moderately differentiated colon adenocarcinoma. n.t., not tested; −, staining undetectable; ±, weak staining; +, strong staining.

DISCUSSION

Using *in situ* hybridisation and immunohistochemistry, we demonstrated that the majority of human colorectal adenocarcinomas tested synthesise the chemokine IL-8. Although a range of human tumour cells lines have previously been shown to secrete chemokines [1, 2], our observations emphasise the high incidence of IL-8 production by colorectal tumour cells *in vivo*. As IL-8 mRNA was, in some samples, more readily detectable than IL-8 protein, this may indicate that a degree of post-transcriptional regulation of IL-8 production is operative in some tumours. The synthesis of tumour necrosis factor α (TNFα) has been reported to be under translational control, mediated via 3′ untranslated sequences in TNFα mRNA [14]. As chemokines are also predominantly inducible pro-inflammatory mediators, a similar mechanism of regulation may apply to IL-8 in these tissues. Alternatively, the anti-IL-8 monoclonal antibody may not bind optimally to IL-8 in some formalin-fixed, paraffin-embedded tissues. An additional explanation might be that IL-8 is rapidly secreted or metabolised in some tumours.

In order to generate a stable chemotactic gradient during an inflammatory response, C–C chemokines such as the macrophage inflammatory protein (MIP)-1β are able to bind to endothelial cell surface proteoglycans [15]. In the present study, IL-8 was detected in endothelial cells, but antibody localisation was not noticeably associated with the plasma membrane. A possible explanation is that the epitope recognised by the monoclonal antibody was masked by binding to cell surface proteoglycan. Alternatively, intracellular levels of IL-8 may exceed those bound to cell surface structures and hence be more readily detectable by immunocytochemistry.

Despite the presence of IL-8 in most tumours in this study, neutrophil infiltration is not a common feature of colorectal tumours. The chemotactic response of neutrophils to IL-8 follows a bell-shaped dose–response curve [2], and production of high levels may be supra-optimal for neutrophil chemoattraction. Anti-IL-8 auto-antibodies have been detected in normal human sera, which block binding of IL-8 to neurophils and bind essentially all free IL-8 in serum [16]. The presence of such blocking antibodies in patients with colorectal tumours might also explain the relative lack of neutrophil infiltration in the presence of significant amounts of IL-8. However, it was not possible to quantify IL-8 production by tumour cells in the present experiments, and the possibility remains that high levels of IL-8 were not secreted by tumour cells, despite its ready detection in the cytoplasm.

For a high proportion of colorectal tumours to synthesise IL-8, this chemokine might be expected to subserve a role in enhancing tumour growth or metastasis [17]. As well as attracting neutrophils to sites of inflammation, IL-8 can also induce them to secrete granule-associated enzymes, such as gelatinase [18], which may facilitate tumour invasion or metastatic spread. In addition, macrophage-derived [17] or tumour-derived IL-8 [19] can be angiogenic, which would enhance the blood supply to the tumour. In this context, it has recently been shown that human colorectal carcinomas, particularly those that are poorly differentiated, exert an angiogenic effect *in vivo* [20].

Another member of the C–X–C chemokine subfamily, known as gro or melanoma growth stimulating activity (MGSA), has, as its name suggests, autocrine growth activity for cultured human melanoma cells [21]. It has also been shown that IL-8, as well as enhancing the metastatic spread of human melanoma cells in nude mice, can act as an autocrine growth factor for this tumour cell type [12]. Whether this might be true for human colorectal carcinoma cells is unclear. However, if these tumours are able to consume much of the IL-8 that they produce, this would explain the relatively sparse neutrophil infiltrate and the inability to detect IL-8 immunohistochemically in significant amounts outside the tumour cell cytoplasm. Such a function of IL-8 would require the expression of IL-8 receptors on the tumour cells themselves and future work will address this.

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