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Expression of Chemokine Receptor CXCR6 in Human Colorectal Adenocarcinomas

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Abstract. *Infiltration of inflammatory cells into colorectal adenocarcinomas is considered of importance for tumour progression. Tumour-associated macrophages and T cells are predominant components of the chemokine-guided filtrate of most colorectal tumours. CXCR6 is a chemokine receptor expressed by Th1, Tc, NKT cells and smooth muscle cells. To determine whether CXCR6 is expressed in human colorectal cancer and corresponding normal tissue, we analysed CXCR6 protein expression in 32 surgical specimens. Immunohistochemistry revealed CXCR6 protein predominantly localised in normal epithelial cells and some scattered stromal cells. No or weak expression was found in cancerous tissue. Western blot analysis showed, in 41 % of the cases, a notable suppression of CXCR6 protein ($p<0.05$) in cancerous tissue compared with non-cancerous tissue. Up-regulation was found in 9% of the cases. CXCR6 protein expression, in 25% of the cases, showed no difference between tumour and adjacent normal tissue. Furthermore, 25% of the cases revealed undetectable levels of CXCR6 protein in tumour as well as corresponding normal tissue. The results may reflect one of the immunological features of normal and cancerous colorectal tissue and studies on regulation of CXCR6 are necessary in order to determine its role in colorectal carcinogenesis.*

Colorectal cancer (CRC) is a common cause of death from cancer in Western countries. The major part of CRC is derived from the mucosa and it is clear that genetic alterations in epithelial cells can enter the neoplastic process (1). Leukocyte infiltration of macrophages and T cells is found in human epithelial cancers and expresses a complex network of cytokines and chemokines (2-4). Cytokines, growth factors and angiogenic factors, among others, are released from leukocytes attracted by chemokines into the tumour microenvironment. Tumour-associated leukocytes contribute to growth and spread of the malignancy, but the leukocytes have also been shown to play an antitumourigenic role (2,5,6).

Several CC and CXC chemokine receptors are variably expressed on a number of colonic adenocarcinoma cell lines, but also on colon epithelial cells *in vivo* (7-9). However, the role of many of the chemokines and receptors are unclear. Blocking CXCL1 or its receptor CXCR2 with specific antibodies inhibited the growth of melanoma cells *in vitro* (10). Conversely, the over-expression of CXCL1 (11), CXCL2 or CXCL3 (12) in various melanoma cell lines increased their ability to form colonies in soft agar and their tumourigenicity in nude mice. Furthermore, expression of CXCR2 on certain cells in the presence of persistent autocrine and paracrine stimulation with specific CXC chemokine ligands can promote pre-neoplastic into neoplastic cellular transformation (13).

CXCR6 is a chemokine receptor for the chemokine CXCL16 and is expressed by Th1, Tc, NK, NKT cells, bone marrow plasma cells, myeloma cells and smooth muscle cells (14-17). CXCR6-expressing Th1 cells display tissue-homing potential and are enriched in inflamed tissues, suggesting its novel role in mediation of type I inflammation (15). Interestingly, CXCL16, expressed on monocytes/macrophages, B cells and dendritic cells in the T cell zone of lymph nodes, by sinusoid associated cells of the splenic red pulp (18,19), as well as by human T cells, (15) functions as a cell adhesion molecule for cells expressing CXCR6 (20).

To date, the expression of CXCR6 has not been described in human colorectal cancers. Therefore, we wanted to study if CXCR6 is expressed in the specimens of cancerous and adjacent non-cancerous tissue.

Materials and Methods

Patients and sampling. This study comprised tissue samples, which were obtained from 32 consecutive patients who had undergone surgical resections for primary colorectal adenocarcinomas,

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diagnosed at the Department of Surgury, Ryhov County Hospital, Jönköping, Sweden. This study group comprised 19 males and 13 females. The mean age was 71 years, with a range from 50-90 years, and all tumours were classified according to the Dukes' classification system; stage A (n=4), stage B (n=14), stage C (n=13) and stage D (n=1). Eighteen were located in the rectum and 14 in the colon. From each patient tumour tissue and adjacent normal mucosa (about 5 cm from the tumour) was excised and immediately frozen at -70˚C until analysis.

Protein preparation. Frozen tumour tissue and normal mucosa were thawed, homogenised in ice cold lysis buffer containing PBS (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, pH=7.4) and 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 100 μg/ml phenylmethylsulphonyl flouride (PMSF), 2 μg/ml aprotinin, 1 mM sodium orthovanadate and 1 μg/ml leupeptin. The lysate was placed on ice for 30 minutes and then centrifuged at 13,000 g for 10 minutes. The protein content of the supernatant fluid was determined for each sample using the Lowry assay (21).

Western blot analysis. Proteins (60 μg) in the lysates were separated under reducing conditions by electrophoresis using 10% SDS-PAGE (polyacrylamide gel electrophoresis) (21). Separated proteins were transferred onto nitrocellulose membranes (Amersham, Buckinghamshire, UK) in a transblot electrophoretic transfer cell (Bio-Rad Laboratories, Hercules, CA, USA). After transblotting, the blots were blocked for two hours with 5% (wt/vol) non-fat dry milk dissolved in TTBS (1xTTBS: 20mM Tris, 150 mM NaCl, pH 7.5, 0.1% Tween 20). The blots were probed for four hours with an affinity-purified rabbit antihuman polyclonal CXCR6 antibody (Prosci Inc, Poway, CA, USA) diluted in TTBS (2 μg/ml) containing 3% (wt/vol) non-fat dry milk. After extensive washing in TTBS, the blots were reincubated for two hours with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotecchnology, Santa Cruz, CA, USA) diluted 1:4000 in TTBS containing 3% (wt/vol) non-fat dry milk. Blots were developed using an enhanced chemiluminescence (ECL) system (Amersham) and exposed to Hyperfilm ECL (Amersham). Loading was controlled by an anti-human monoclonal actin antibody (Santa Cruz Biotechnology). The optical density was measured by densitometry and the result is shown as relative expression for tumour *versus* normal mucosa.

Immunohistochemistry. Ten of the 32 tumour samples were available for immunohistochemical staining to study the cell type origin of the CXCR6 expression. Staining for CXCR6 was performed using a standard protocol on slides from formalin-fixed paraffin-embedded tissue blocks. The incubation of rabbit anti-human polyclonal CXCR6 antibody was followed by incubation with a biotinylated goat anti-rabbit IgG (Dakopats, Glostrup, Denmark). For anti-CXCR6 antibody blocking, serving as negative control, the antibody was preincubated for 1 hour at 37˚C with 10 times excess of the antigenic CXCR6 peptide (Prosci). Avidin-biotin peroxidase complexes (ABC Elite Kit, Vector Labs, Burlingame, CA, USA) were added, followed by visualization with 3.3'-diaminobenzidine tetrahydrochloride (Vector Labs). The slides were then counterstained with Mayer's hematoxylin.

Statistical analysis. Differences of CXCR6 protein between tumour and paired normal tissues were examined by Wilcoxon signed rank test. The data were subjected to computerized statistical analysis using the SPSS for Windows version 6.1 and the results were considered significant at the level $p<0.05$.

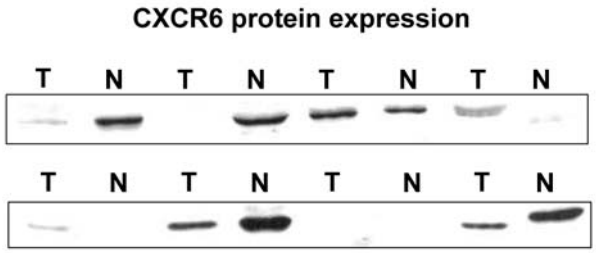

Figure 1. *Western blot analysis of CXCR6 (43 kDa) protein expression in 8 representative specimens of human colorectal cancer in two separate experiments. T, tumour tissue; N, matched normal tissue.*

Results

Western blot analysis. Western blot analysis of CXCR6 protein in human colorectal tumour tissue was pair-wise related to normal mucosa from 32 examined surgical specimens. Probing of tissue proteins immobilized on nitrocellulose membranes with anti-human CXCR6 antibody and subsequent semi-quantitative evaluation by densitometry showed a relative expression (tumour *vs* normal mucosa) in 13 cases < 1 ($p<0.05$), in 8 cases =1 and in 3 cases > 1. No CXCR6 expression was observed in 8 cases. A representative immunoblot prepared from 8 patients with matched normal tissue CXCR6 expression is shown in Figure 1. Actin expression was used to demonstrate protein loading (data not shown). Expression of the CXCR6 protein levels in all analyzed tissues did not correlate with clinicopathological characteristics such as Dukes' stage and localisation (data not shown).

Immunohistochemical findings. Non-cancerous tissue (resected outside the tumour tissue) showed immunoreactivity of CXCR6. CXCR6 was predominantly localised in normal epithelial cells and also in some scattered stromal cells in the lamina propria, with the morphological characteristics of lymphocytes and fibroblasts. Overall, in normal epithelial cells, CXCR6 protein was detected where the intensity of staining varied from weak to strong cytoplasmic expression. No staining was observed in carcinoma cells in cancerous tissue. In the surrounding stroma a few stromal cells were stained weakly, Figure 2.

Discussion

In an ongoing effort to clarify colorectal carcinogenesis with special reference to the underlying immunological mechanisms, we analyzed the protein expression of the

chemokine receptor CXCR6. Using Western blot analyses, we demonstrated that there was a notable suppression of CXCR6 protein in 41% (13/32) of the colorectal tumours compared with adjacent normal mucosa. Nine percent (3/32) showed up-regulation and 25% (8/32) showed no difference between tumour and adjacent normal tissue. Twenty-five % (8/32) of the cases revealed undetectable levels of CXCR6 protein in tumour as well as corresponding normal tissue.

We used immunohistochemistry to discriminate the origin of the CXCR6 expression. This investigation revealed CXCR6 protein expression mainly in normal epithelial cells and in sporadic lymphocytes and fibroblasts located in lamina propria. In cancerous tissue, no/weak staining could be observed in carcinoma cells as well as in stromal cells.

In recent years it has become clear that chemokines and their receptors are involved in the promotion or inhibition of tumour development (2,13). Attention has been focused on the hypothesis that macrophages and cancer cells can secrete cytokines and provide directional stimulus for the movements of tumouricidal lymphocytes. In some cases the tumour infiltrating macrophages appear to be able to produce angiogenic factors and proteases that favour tumour progression and metastasis (22). CXCL16 is a newly-discovered CXC chemokine, which exists both in a transmembrane and soluble form. Membrane CXCL16 is expressed by macrophages, B cells and dendritic cells. Soluble CXCL16 has been shown to induce chemotaxis of Th1, Tc1 and NK T cells, which express the functional receptor CXCR6.

In the gastrointestinal tract, the intestinal mucosa regulates trafficking of leukocytes into and out of the lamina propria adjacent to the epithelium. This may reflect production of chemokines by intestinal epithelium (23).

In the present study, CXCR6 protein was expressed in 75% of the cases including normal and tumour colorectal tissue. In 41% of the cases the level of CXCR6 protein was higher in normal tissue compared with adjacent tumour tissue. One may speculate that, in normal tissue, the epithelial chemokine receptor CXCR6 is able to enrich the mucosa with antigen-presenting cells by direct binding to membrane-anchored CXCL16. The down-regulation of CXCR6 seen in tumour tissue could be a strategy by tumours to escape confrontation with tumour infiltrating leukocytes such as antigen-presenting cells.

The control of CXCR6 expression in the gastrointestinal tract is not understood. In this study we were able to demonstrate that 25% of cases showed no CXCR6 expression in normal and tumour tissues. A possible explanation of the absence of CXCR6 protein expression might depend on mutations in still unknown CXCR6-regulating genes. Moreover, the regulating process may require input from multiple signal transduction pathways.

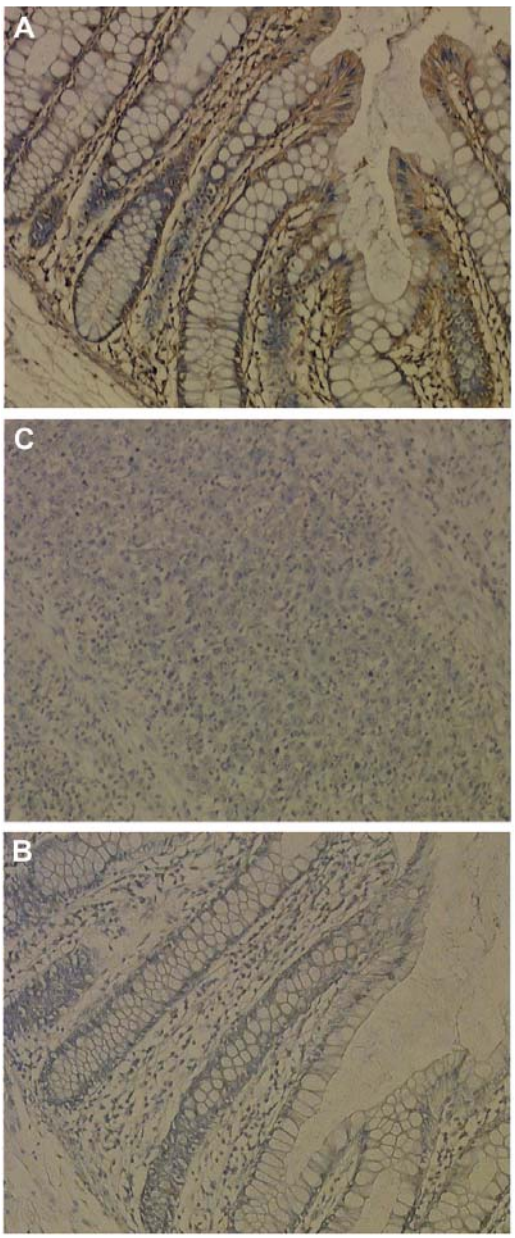

Figure 2. *Representative immunohistochemical staining of CXCR6 protein in human colorectal cancer. (A) CXCR6 expression is present to a large degree in normal epithelial cells and to a low extent in cells located in lamina propria. (B) Negative control. (C) Cancerous tissue showing weak staining of CXCR6.*

Cellular defects in these signalling pathways can differently promote the expression of CXCR6 through the loss of pre- and post- transcriptional regulatory mechanisms.

In summary, the major observations in this study are: (i) CXCR6 protein expression in human cancerous and adjacent normal colorectal tissue and (ii) in a subset of the cases, there is a notable suppression of CXCR6 in

cancerous tissue compared with corresponding normal tissue. The present data may add to our understanding of immunological features in colorectal cancer. The role of CXCR6, by its suggested cell adhering activity in the colon and rectum, remains to be seen. Further investigations are needed to clarify the precise role of CXCR6 in colorectal carcinogenesis.

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