Expression of Interleukin-17 in Human Colorectal Cancer

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Abstract. The proinflammatory cytokine IL-17 plays a potential role in T-cell mediated angiogenesis and promotes tumourigenicity of human cervical cancer. The objective of this study was to determine whether IL-17 protein level is altered in colorectal tumours (n=74) compared with paired normal mucosa and in plasma from patients (n=61) with colorectal cancer (CRC) compared with a healthy group (n=78). Analyses by ELISA showed that IL-17 protein was undetectable in 48.6% of the patients with cancer, as well as corresponding normal tissue which may in part reflect an individual difference. No significant difference was observed regarding IL-17 protein levels between cancer and matched normal tissue or in plasma between patients and the healthy group. Immunohistochemistry (n=20) revealed heterogenous immunoreactivity in 65% of the cases. The results of this study suggest that IL-17 plays a minor or partial role in CRC.

Infiltration of inflammatory cells into colorectal cancer (CRC) tissue is considered of importance for tumour progression (1). There is evidence that proinflammatory cytokines, which can be produced by various tumour cells and tumour-associated leukocytes, play an important antitumourigenic role, but have also been shown to contribute to growth and spread of the malignancy (2, 3). Examples are interleukin-1 (IL-1) and IL-6 which induce proliferation and prolonged cell survival (4-6). The proinflammatory cytokine IL-17 is predominantly produced and secreted by activated CD4 T-cells (7) but data in humans have subsequently demonstrated that CD8 T-cells can also produce IL-17 (8). In addition to the proinflammatory response, IL-17 is able to regulate tight junction formation (9) and has an anti-mitotic effect on the intestinal epithelial cells (10). IL-17 promotes tumourigenicity of human cervical cancer and is associated with an increased level of IL-6 which correlates well with the invasiveness of cervical tumours (11, 12). Moreover, IL-17 has been shown to be expressed in a considerable proportion of ovarian cancers and promotes tumour angiogenesis (13). Numasaki et al., reported that IL-17 plays a crucial role in T-cell mediated angiogenesis (14) and it has recently been suggested that IL-17 may promote angiogenesis by enhancing vascular endothelial growth factor (VEGF) (15). A correlation between vascularity and invasive behaviour in CRC has also been demonstrated (16). Furthermore, it has been shown that VEGF expression in the early premalignant stage of colorectal tumour progression is significantly up-regulated compared to normal colorectal tissue (17). These reports indicate a role of IL-17 in the promotion of tumour progression. However, to our knowledge little is known about the expression and the pathophysiological role of IL-17 in human CRC.

In this study IL-17 protein expression in specimens of cancer and adjacent non-cancer tissues and levels of IL-17 protein in plasma from colorectal cancer patients were investigated.

Materials and Methods

Patients and tissue sampling. This study utilized tissue samples, which were obtained from 74 patients who underwent surgical resections for primary colorectal adenocarcinomas diagnosed at the Department of Surgery, Ryhov County Hospital, Jönköping, Sweden. Sporadic tumours from 40 male and 34 female subjects with a mean age of 70 years (range 36-93) were collected and classified according to Dukes’ classification system: stage A (n=11), stage B (n=30), stage C (n=29) and stage D (n=4). The tumours were localized in the colon (n=34) and rectum (n=40). From each patient tumour tissue and adjacent normal mucosa (about 5 cm from the tumour) were excised and immediately frozen at –70°C until analysis.

The study was approved by the Ethics Committee of our hospital and all patients gave informed consent.

Tissue protein preparation and ELISA. Frozen tissue samples, which were obtained from 74 patients who underwent surgical resections for primary colorectal adenocarcinomas diagnosed at the Department of Surgery, Ryhov County Hospital, Jönköping, Sweden. Sporadic tumours from 40 male and 34 female subjects with a mean age of 70 years (range 36-93) were collected and classified according to Dukes’ classification system: stage A (n=11), stage B (n=30), stage C (n=29) and stage D (n=4). The tumours were localized in the colon (n=34) and rectum (n=40). From each patient tumour tissue and adjacent normal mucosa (about 5 cm from the tumour) were excised and immediately frozen at –70°C until analysis.

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Key Words: Interleukin-17, protein expression, colorectal cancer.
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 mg/ml phenylmethylsulphonyl fluorid, 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 xg for 10 minutes. Protein content of the supernatant fluid was determined for each sample using the Lowry assay (18).

The IL-17 protein levels of cancer and paired normal tissues from 74 patients were measured using established commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Europe, UK). The IL-17 protein level was expressed as picograms per milligram of protein (pg/mg). All analyses were performed in duplicate and the mean values were used for statistical calculations.

**Plasma samples and ELISA.** Sixty-one of the patients (31 rectal cancers and 30 colon cancers) were available for plasma collection. Venous blood was collected before surgery and separated by centrifugation within 1 hour.

In order to establish the IL-17 level among healthy individuals 78 control subjects were selected from volunteer blood donors at Ryhov County Hospital. The mean age was 60 (55-67) years and included 41 males and 37 females. All plasma from patients and control subjects was stored at –70ºC until IL-17 was measured by ELISA (R&D Systems Europe, UK) following the manufacturer’s instructions.

**Immunohistochemistry.** Twenty out of the 74 tumour samples were available for immunohistochemical staining to study the cell type origin of the IL-17 expression. Staining was performed using a standard protocol on 4 µm sections from formalin-fixed paraffin-embedded tissue blocks. Endogenous peroxidase activity was quenched by treatment with 3% hydrogen peroxide for 5 min followed by incubation with 5% blocking bovine serum albumin solution. Sections were subsequently incubated with a primary rabbit anti-human polyclonal antibody (Santa Cruz Biotechnology, USA) in appropriate dilution (1:100) overnight at 4ºC. After rinsing in tris-buffered saline, sections were incubated with secondary biotinylated bovine anti-rabbit IgG (Santa Cruz Biotechnology). Avidin-biotin peroxidase complexes (Dako Cytomation, Denmark) were added followed by visualization with 3,3'-diaminobenzidine tetrahydrochloride (Dako Cytomation). All sections were counterstained with Mayer’s haematoxylin (Histolab Products AB, Sweden).

For negative control, non-immune rabbit isotypic IgG (Santa Cruz Biotechnology) was added instead of the primary antibody.

**Statistical analysis.** Data are presented as mean values ± standard error of the mean (SEM). Differences of IL-17 protein expression between tumour and paired normal tissues were examined by the Wilcoxon signed rank test. Statistical analysis was performed using the SPSS for Windows computer package (Rel. 11.5, 2002, Chicago: SPSS Inc.). Results were considered significant at a level of p<0.05.

**Results**

**Levels of IL-17 in tissue and plasma.** IL-17 protein concentration was measured by ELISA in protein-lysates of CRC tissues and matched normal tissues from 74 patients. IL-17 protein expression was undetectable in 48.6% (36/74) of the CRC cases, as well as in corresponding normal tissue. Evaluation of the relative expression (tumour versus normal tissue) showed 31.1% (23/74) up-regulation and 20.3% (15/74) suppression. The levels of IL-17 protein in cancer tissue (6.9±1.35 pg/mg) showed no significant difference in comparison with normal tissue (7.1±1.93 pg/mg), (p=0.421) and are shown in Figure 1 which is given as scatter graph.

IL-17 in plasma was detected in 12 out of 61 in the CRC group and 8 out of 78 in the healthy donor group and the range was 2.1-73.8 pg/ml and 8.8-54.1 pg/ml, respectively. There was no statistical difference in IL-17 levels between these groups and no relation between IL-17 levels in tissue and plasma (data not shown).

**Location of IL-17 expression in CRC tissue.** To identify the localization of IL-17 protein in CRC tissue immunohistochemical analysis was performed in 20 CRC cases. The resection border reflects normal tissue and haematoxylin staining revealed stromal cells which were identified by their morphological appearance. Immuno-reactivity was detected in 13 out of 20 (65%) cases and showed heterogenous and focal staining of IL-17. IL-17
immunoreactivity was localised in cancer and normal epithelial cells but also in stromal cells with the morphological characteristics of lymphocytes and fibroblasts (Figure 2). Overall, an intensity of cytoplasmic staining varying from none to weak and occasionally strong was noted. Negative controls had no immunoreactivity.

The levels of IL-17 protein in all the analyzed tissue and plasma samples from the CRC patients did not correlate with clinical characteristics such as age, gender, location and Dukes’ stage (data not shown).

**Discussion**

Elevation of proinflammatory cytokines, such as IL-1β and IL-6 is considered to be associated with colorectal cancer (4, 19). In *in vitro* assay, IL-17 induces IL-1β from macrophages and has been shown to stimulate epithelial and fibroblastic cells to secrete IL-6 (20, 21). Recently, IL-17 has been demonstrated to increase the production of active metalloproteinase MMP-9 (22). The role of metalloproteinases in the progression of human malignancies has been well documented in numerous reports (23, 24). Moreover, IL-17 promotes angiogenesis and tumour growth (14). These circumstances may implicate a role for IL-17 in colorectal carcinogenesis.

In the current study an ELISA assay was used to determine the level of IL-17 protein in CRC and paired normal tissue. No significant difference regarding IL-17 protein expression was observed between cancer and matched normal tissue in the patient samples. Moreover, IL-17 protein could not be detected in 48.6% of the patients in either cancer or paired normal tissue. The discrepancy may in part reflect individual differences. To examine whether IL-17 protein was expressed in tumour and normal epithelial or stromal cells in CRC immunohistochemistry was carried out. Staining revealed heterogenous immunoreactivity with no specific difference in cancer and normal epithelial or stromal cells.

Circulating cytokines such as IL-1 and IL-6 are associated with CRC (6, 25, 26) as is the angiogenetic factor VEGF (27). While searching for tumour markers, the levels of IL-17 in plasma from CRC patients were also studied. However, the levels of IL-17 in plasma from the patients were not significantly different than those in the healthy control group.

Until today there has been little information available about IL-17 in cancer, especially in human CRC. Our results may suggest that IL-17 plays a minor or partial role in CRC for instance in connection with T-cell mediated angiogenesis and IL1-β/IL-6 derived tumour growth.
Further investigations are essential to clarify whether cytokine IL-17 is involved in colorectal carcinogenesis and its clinical relevance. The results presented here comprise the initial stage of a forthcoming study on the immunological features of CRC in our laboratory.

Acknowledgements

This work was supported by grants from the County Council of Jönköping, Sweden, Health Research Council in the South-East of Sweden and the University College of Health Sciences, Jönköping, Sweden. We thank Elisabeth Slänemyr for excellent technical assistance.

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