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Immunophenotype of Isolated Tumour Cells in the Blood, Bone Marrow and Lymph Nodes of Patients With Gastric Cancer

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Immunophenotype of isolated (disseminated or circulating) tumour cells (ITC) in the blood, bone marrow and lymph nodes were studied in patients with gastric cancer. Coexpression of metalloproteinases inducer (EMMPRIN), chemokine receptors (CCR6, CXCR4) and adhesion molecules (Ep-CAM, CD44) was determined on cytokeratin positive (CK^+) cells in $CD45^-$ cell population sorted out from the blood and/or bone marrow. Eight cytospin samples of blood and 69 samples of bone marrow containing CK^+ cells from patients with gastric cancer were included into study. Expression of EMMPRIN and CCR6 were noted in a half of CK^+ samples (of blood/bone marrow) whereas the expression of CXCR4 and Ep-CAM was much lower. Analysis of paired data of these determinants expression on CK^+ cells showed no association between them. Expression of EMMPRIN, Ep-CAM, CCR6, CCR7, CXCR1, and CXCR4 on ITC in lymph nodes was determined by flow cytometry. In 18 lymph nodes (out of 36 assayed) CK^+ cells were found. The expression of CCR6 and Ep-CAM on CK^+ cells was observed in almost all studied lymph nodes, CXCR1 – in half of them. The expression of EMMPRIN and CCR7 cells was lower. These results suggest that ITC of gastric cancer express variably several molecules that may be involved in metastasis formation.

Introduction

Determination of tumour cell-associated antigens, including different epitopes of cytokeratins (CK), is commonly used for detection of ITC in the blood, bone marrow and lymph nodes [4, 6, 8, 9, 28, 29, 32]. The formation of a micrometastasis is a complex phenomenon which starts from the migration of cancer cells from the primary tumour and only very small percentage of isolated tumour cells (0.05%) in the blood stream form the metastatic lesion [7]. Expression of chemokine receptors, metalloproteinases (MMPs), metalloproteinase inducers (EMMPRIN) and adhesion molecules facilitates the process of micrometastasis formation [1, 5, 6, 12, 16, 19]. The cell cycling, trafficking and homing are regulated by many mediators including stromal cell derived factor (SDF-1). Expression of SDF-1 on endothelial cells and the presence of chemokine receptor for SDF-1 (CXCR4) on tumour cells may be involved in the release of cancer cells from the primary tumour and their migration via the blood vessels to the tissues [1, 13, 25]. Moreover, expression of CXCR4 on tumour cells and the presence of SDF-1 on bone marrow stromal cells contribute to homing of cancer cells in the bone marrow [1, 11]. Metastases of gastric cancer in the lymph nodes are associated with the expression of CCR7 on tumour cells [1]. Among various chemokine receptors regulating the cell migration and trafficking, the expression of CCR6 found on cancer cells may explain the formation of metastases in the liver observed in various cancer, including gastrointestinal tumours. Expression of CCL20 (CCR6 ligand) on normal liver cells attracts CCR6 positive cells to home in the liver [5, 25]. The process of cancer cells migration and spread are also associated with the expression of adhesion molecules on tumour cells (e.g. epithelial cell adhesion molecules, Ep-CAM or CD44) [22, 27, 31, 33]. The enhanced expression of Ep-CAM in gastric cancer is associated with better prognosis [27].

The present studies were designed to determine the expression of EMMPRIN, Ep-CAM, CCR6, CCR7, CXCR1, CXCR4, CD44 on CK^+ cells present in the blood, bone marrow and in lymph nodes of patients with gastric cancer.

Patients and Methods

Patients

Bone marrow and peripheral blood samples were obtained from 198 patients with gastric cancer after providing written informed consent. The clinical diagnosis was confirmed by histopathological examination. The peripheral blood (20 ml) and bone marrow samples (1 ml) from iliac crest biopsies were collected in EDTA-containing plastic tubes under general anaesthesia prior the surgery.

Isolation of CD45$^-$ cells

The population of CD45$^-$ cells from peripheral blood and bone marrow was obtained as previously described [23]. Population of non-hematopoietic (CD45$^-$) cells containing presumptive tumour cells was used for preparation of slides (4–6 from each sample). One slide (from the blood and bone marrow) was fixed with an ethanol and acetone mixture (1:1 vol), then stained with phycoerythrin (PE) conjugated A45-B/B3 monoclonal antibody (mAb) that recognizes epitopes of cytokeratins (CK) 8, 18, 19, (Micromet GmbH, Germany) (20 µl mAb/50 µl of PBS, per slide, 30 min incubation at room temperature). After washing with phosphate buffered saline (PBS), the cover slides were placed and the slides examined under BX60 fluorescent microscope (Olympus, Tokio, Japan) and documented with DP10 camera (Olympus). The remaining slides were kept in –70°C until testing. Approximately 300 cells were examined and slides were regarded as positive when at least 3 CK$^+$ cells were recorded. Only the slides previously identified as containing CK$^+$ cells were used. Double staining with PE-conjugated anti-cytokeratin mAb followed by anti-CXCR4, CCR6 (R&D, Germany), EMMPRIN (Santa Cruz, Biotechnology, Santa Cruz, CA), Ep-CAM (Ber-Ep4, Santa Cruz Biotechnology), CD44 (Becton-Dickinson, San Jose, CA) mAbs conjugated with fluorescein (FITC) was performed.

Lymph nodes of 36 patients with gastric cancer were obtained from surgical specimens of resected gastric cancer. The mechanically obtained single cell suspension was adjusted to 1 x 10^6 cells/ml, washed with PBS and stained (10^5 cells/tube) with mAbs against the following determinants: EMMPRIN, Ep-CAM, CCR6, CXCR4, CCR7 and CXCR1 (R&D, Germany for the last 2 mAbs). After incubation (30 min at room temperature), washing with PBS cells were fixed, permeabilised (Cytofix/Cytoperm, Perm/Wash buffer, Becton-Dickinson) and stained with PE-conjugated anti-CK mAb. Isotype matched control of mouse IgG1 and IgG2a, IgG2b (R&D, Santa Cruz) was used. After washing the cells were suspended in PBS. For analysis by flow cytometry (FACSCalibur, Becton-Dickinson, Immunocytometry Systems, San Jose, CA) 20 000 events from each sample were acquired. The suspension of lymph nodes cells containing more than 3% of CK$^+$ cells was regarded as positive and analysed.

Statistics

The association between expression of given determinants was assayed based on Wilcoxon matched pairs test from GraphPad Instat (GraphPad Software, Inc. San Diego, California, USA) .

Results

Expression of EMMPRIN, Ep-CAM, CD44 and chemokine receptors (CCR6, CXCR4) was determined on CK$^+$ in the smears of CD45$^-$ cells isolated from the blood (n=8) and bone marrow (n=69) of 198 patients with gastric cancer. However, the actual number of determinations was usually lower due to the absence of CK$^+$ cells in individual slides.

Expression of CD44, CXCR4 and Ep-CAM was observed in 10.6%, 23.3% and 39.4% of CK$^+$ samples, respectively (Fig. 1). Proportion of CCR6 and EMMPRIN positive cells was high and observed on approximately half of CK$^+$ samples (Fig. 1, Fig. 2A, B). The analysis of paired data (28 sets of samples) did not show the statistically significant associations between these determinants expression. Comparison of immunophenotype of tumour cells in 7 samples of blood and bone marrow from the same patients showed no differences in the expression of these determinants.

Flow cytometry analysis of cells from 18 lymph nodes of 36 patients showed the presence of EMMPRIN and CCR7 on CK$^+$ cells in 33.3% and 38.8% lymph nodes, respectively. Expression of CXCR4 and CXCR1 was similar and observed in approximately half (55.5%) samples of lymph nodes. The expression of CCR6 and Ep-CAM was observed in majority of lymph node CK$^+$ cells (Fig. 3).

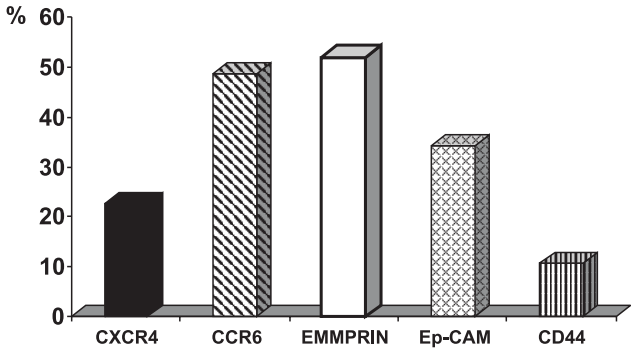

Fig. 1. Expression of different determinants on CK$^+$ cells in the peripheral blood and/or bone marrow samples of patients with gastric cancer.

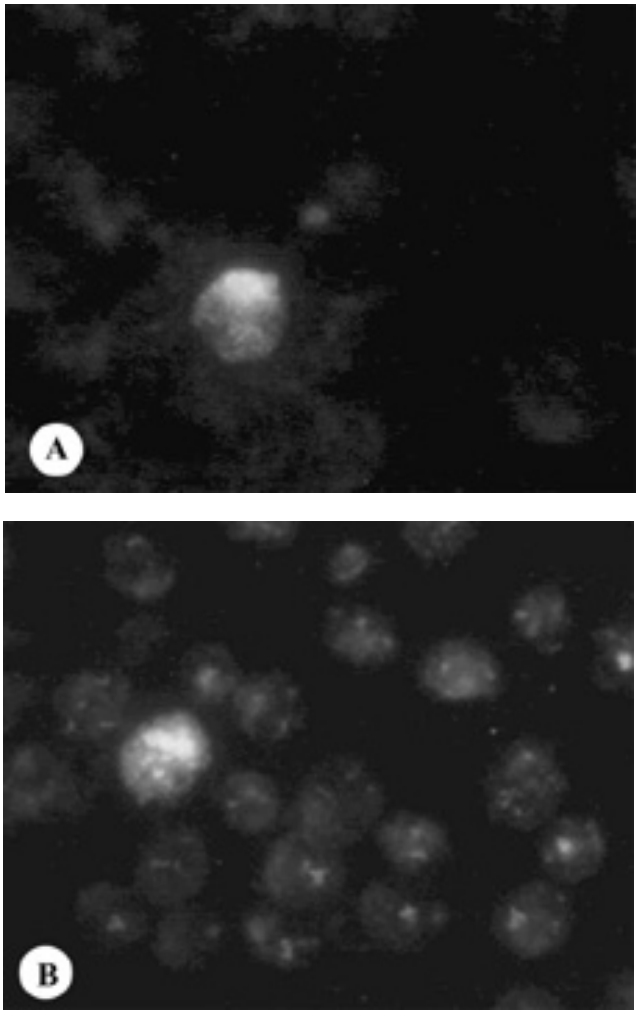

Fig. 2. The presence of Ep-CAM (A) and EMMPRIM (B) on CK^+ cells in bone marrow samples. Ethanol/methanol fixed slides were stained with PE-labelled anti-CK mAb followed by FITC conjugated anti-EpCAM and anti-EMMPRIN mAb. CK – orange fluorescence, Ep-CAM, EMMPRIM – green fluorescence. Photo taken by DP camera. Magn. 400 ×.

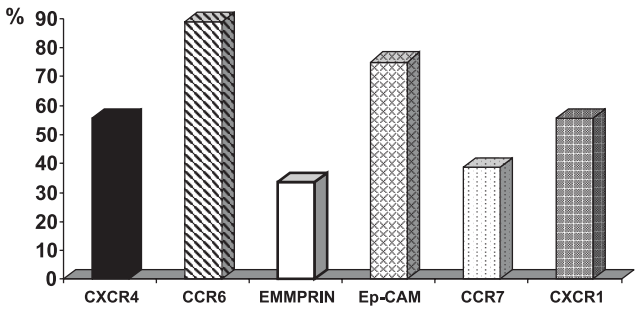

Fig. 3. Expression of different determinants on CK^+ cells in the lymph nodes of patients with gastric cancer.

Discussion

The formation of metastases is a result of cancer cells release from the primary tumour, survival in the blood stream, homing and proliferation in target organ. In gastric cancer spread of tumour cells occurs via lymphatic and blood circulation. The immunophenotype of microdisseminated tumour cells in different cancers, including gastric cancer, is not fully determined.

The CK^+ cells, presumptive tumour cells, circulating in the blood stream and/or present in bone marrow showed the expression of EMMPRIN and CCR6 in about half of studied samples. Expression of CXCR4, CD44 and Ep-CAM was much lower. The analysis of paired data showed independent expression of studied determinants, but no differences in their expression were seen when the blood and bone marrow samples were compared. The immunophenotype of CK^+ gastric cancer cells from lymph nodes was different as compared to CK^+ cells from the bone marrow and blood samples as expression of Ep-CAM, CCR6, CXCR4 was higher in the former suggesting their role in homing to the lymph nodes. In contrast, an increased proportion of EMMPRIN positive cells was observed in the bone marrow.

The metalloproteinases (MMPs) reacting with type IV collagen, laminin, fibronectin are associated with invasion of the tumour into surrounding tissues [19, 20, 21, 26]. Gastric and oesophageal cancer cells showed expression of MMP-2 (gelatinase A), MMP-9 (gelatinase B) and MMP-7 (matrilysin) that are involved in remodellling of extracellular matrix and regulate angiogenesis [6, 15, 20]. Moreover, cancer cells express the inducer of MMPs (EMMPRIN) [3, 19, 20]. In colorectal cancer the induction of MMPs production was observed by metastatic cells [26]. Our observation of high frequency of EMMPRIN expression on CK^+ cells in blood/bone marrow probably represents the same phenomenon in gastric cancer. The lower frequency of EMMPRIN expression on tumour cell in lymph nodes might be associated with different mode of spread to the lymphatic tissue.

In hematogeneous dissemination an increased expression of adhesion molecules (e.g. Ep-CAM) is critical for the interaction of cancer cells with endothelial cells during intra- and extravasation [6, 24]. The gastrointestinal tumour cells express high level of Ep-CAM [26, 31] which is used for detection of cancer cells in the bone marrow [14]. It is interesting that in gastric cancer patients a better survival is associated with expression of Ep-CAM on tumour cells and its loss with shorter survival [27]. In lung cancer the expression of Ep-CAM on tumour cells increased along the involvement of regional lymph nodes [24]. Our observation of high frequency of Ep-CAM expression on CK^+ cells from the lymph nodes is in keeping with these data.

Different chemokines (e.g. CXCL8, CXCL1) and their receptors (CXCR1-CXCR3, CXCR4) are expressed on gastric cancer cells [1]. The expression of CCR7 correlated

with formation of lymph nodes metastases and poorer prognosis in gastric cancer [16]. Activation of CCR7 resulting in polymerisation of intracellular actin and increased motility of cell facilitates the formation of metastases in lymphatic tissue [16, 17, 18]. In our study the expression of CCR7 was noted in about 40% of lymph nodes samples.

The expression of ligand for CCR6 (CCL20) on liver cells is associated with chemotaxis of $CCR6^{+}$ cancer cells to liver and forming metastases [5, 25]. The high expression of CCR6 on pancreatic tumour cells may be associated with a high aggressiveness of this tumour [12]. However, the metastatic squamous carcinoma cells of head and neck show down-regulation of CCR6 and up-regulation of CCR7 and the loss of CCR6 is associated with the lymph nodes metastases [30]. It is in contrast to our observations as almost all tumour cells in the lymph nodes expressed CCR6 and less than a half CCR7. This may implicate that homing of metastatic cells to the lymph nodes may be differently regulated in various types of cancer. The expression of CXCR4 and interaction with SDF1 is associated with homing of tumour cells, e.g. breast cancer, melanoma of the skin in bone marrow and lymph nodes, ovarian cancer spread into peritoneum [1]. The present study indicates lower expression of CXCR4 on tumour cells in the blood/bone marrow than in lymph nodes pointing out to the role of this receptor in homing gastric cancer cells also to the lymphatic tissue. The presence of CXCR4 on oesophagus cancer is associated with lymph nodes involvement, bone marrow micrometastases and reduced overall survival [11].

The choice of different markers used in the present study was dictated by their presumptive or documented role in the formation of metastases in distant organs. Our results showed the variable expression of the adhesion molecules, metalloproteinases inducer and some of chemokines receptors on tumour cells present in bone marrow and lymph nodes of gastric cancer patients. Hence, this study failed to define a common immunophenotype of ITC but may suggest that some of these determinants are differently involved in the regulation of metastasis formation in distant organs. This is compatible with a view that phenotypic and genetic differences between primary and metastatic tumours play a role in the metastasis formation [6] hence the use of multiple markers may be important for ITC detection.

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