Decreased Migration of Myeloid Dendritic Cells through Increased Levels of C-Reactive Protein

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Abstract. Background: In head and neck squamous cell carcinoma (HNSCC), a variety of immunomodulatory mediators contribute to strongly impaired immune functions. The secretion of C-reactive protein (CRP) by HNSCC cells and its influence on human myeloid dendritic cells (MDC) was investigated. Materials and Methods: The CRP levels were analyzed using photometric methods and real-time PCR. The MDC were isolated from peripheral blood by 'magnetic bead separation' and incubated with different CRP concentrations. The CRP isoforms were analyzed by native PAGE (polyacrylamide gel electrophoresis). The cells were analyzed using migration assays and flow cytometry. Results: HNSCC cell lines were able to autonomously express C-reactive protein. Pentameric CRP triggered the down-regulation of chemokine receptor CCR5 and led to a decreased migration of human MDC. Conclusion: CRP appeared to be a modulator of the migration activity of human MDC. The functional modulation of immune cells represents a crucial immune escape mechanism of human carcinomas.

Head and neck squamous cell carcinoma (HNSCC) is one of the most frequent tumors in the world occurring almost exclusively among middle-aged tobacco and alcohol heavy users (1-3). Various immunomodulating mediators within the HNSCC microenvironment have been reported to contribute to strongly impaired immune functions which allow the tumor to escape from efficient antitumor immune responses (3-5).

Besides immunosuppressive and tumor-promoting cytokines, it has recently been shown in non-small lung cancer that serum levels of C-reactive protein (CRP) were strongly associated with tumor characteristics. High values of CRP correlated with an inability to achieve complete resection of tumor tissue (6). As an acute phase reactant, highly increased levels of CRP have been found in response to infections or different malignant diseases. CRP is generally regarded as an anti-inflammatory molecule active in innate immunity and tissue homeostasis (7). Expression of CRP has been demonstrated in the liver, the kidney, the respiratory tract, the thymus, in smooth muscle cells, and in neuronal cells (7-10). Furthermore, increased levels of CRP have been associated with increased metastasis and tumor progression of various kinds of human carcinomas, but the origin of tumor CRP as well as its immunosuppressive capacity is still unclear (11, 12).

The secretion of CRP by HNSCC cells was investigated and its influence on human myeloid dendritic cells (MDC), which have been shown to infiltrate human solid tumor tissues of HNSCC (13-15), was analyzed.

Materials and Methods

Cell culture. Permanent HNSCC cell lines BHY (Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, (16)), PCI-1 and PCI-13 (hypopharyngeal cancer, Pittsburgh Cancer Institute, PA, USA) were cultured in DMEM-medium (Dublecco’s modified Eagle’s medium, Gibco, NY, USA) supplemented with 10% FCS, 1 mM glutamine and 0.1 mM sodium pyruvate.

Quantification of CRP. The cell free supernatants of the three different HNSCC cell lines (BHY, PCI1 and PCI13) were subjected to photometric CRP quantification after 2x10^6 cells per ml were cultivated for 24 hours at 37°C. The quantification of CRP in patient’s serum and in tumor supernatant was performed in the central laboratory of our hospital. Standard photometric procedures were used to exclusively detect pentameric CRP. Patients selected were between 18 and 65 years old and had all different kinds of HNSCC. After written informed consent has been obtained from all patients, tumor tissue specimens were taken during standard surgical procedures. Simultaneously tumor draining lymph nodes were obtained by neck dissection and examined by a pathologist. Tissue specimens were transported in sterile saline...
and processed immediately after excision. The use of human tissues for research purposes was approved by the ethics committee of the University of Lübeck.

**Flow cytometry.** Surface antigen staining was performed as described previously (17). The cells were stained with fluorescein-5-isothiocyanate (FITC-), and phycoerythrin (PE)-, peridinin-chlorophyll-protein (PerCP)-, conjugated antibodies by incubation on ice for 15 min following by washing with PBS. Fluorescence-labelled monoclonal antibodies against Cluster of Differentiation (CD)-11c, Lin-markers, and HLA-DR as well as chemokine receptor (CCR)-5 were purchased from BD Biosciences (Becton Dickinson Heidelberg, Germany). The samples were analyzed on a FACS Canto (BD Biosciences) and data acquisition was performed using the FACS DIVA software (BD Biosciences). The vital dye propidium iodide (PI) was used in conjunction with Annexin V-FITC staining to identify dead cells or cells which were in an early apoptotic stage (Annexin-V-FITC positive, PI negative).

**Isolation of MDC.** MDC which comprise about 0.5 to 1.0% of the total human peripheral blood mononuclear cells (PBMC) were isolated from human peripheral blood (buffy coats) provided by the blood bank of the University Hospital Lübeck. The healthy blood donors were 18-65 years old and were tested to be negative for allergies. Additional exclusion criteria were manifest infections during the last 4 weeks, fever and medication of any kind. The PBMCs were obtained from theuffy coats by Ficoll-Hypaque density gradient centrifugation as described previously (18). The MDCs were isolated by magnetic bead separation using magnetic labelled anti-BDCA-1 (blood dendritic cell antigen-1) antibodies (Milteny, Bergisch Gladbach, Germany). Apart from myeloid dendritic cells CD1c (BDCA-1) is also expressed on a subpopulation of CD19+ small resting B lymphocytes. Therefore, CD19 MicroBeads were used for the depletion of B cells before enriching BDCA-1+ myeloid dendritic cells as described before (19-21). The isolated cells were analyzed by flow cytometry for surface characteristics and vitality as described previously (21).

**Migration analysis.** Isolated MDC were incubated for 12 hours at 37°C with 10 µg/ml, 50 µg/ml, or 100 µg/ml CRP, respectively. Four separate samples were used for each condition. The ChemoTx system was used to investigate the cellular migration of human MDC with macrophage inflammatory protein (MIP)-1β as a chemotacttractant. The ChemoTx system (NeuroProbe Inc., Gaithersburg, MD, USA) is a family of disposable chemotactic/cell migration instruments. Each ChemoTx instrument includes a specially designed 96 well microplate and a framed filter with the filter membrane bonded to a rigid frame. The microplate provides bottom wells for chemotacttractants and other reagents in cell-migration assays. No top wells are required because the proprietary framed filter is coated with a hydrophobic mask that confines each cell-suspension sample to its site on top of the filter. The 96 sites on the filter correspond to the 96 wells in the microplate. The cell suspension is pipetted directly onto the sites on the top side of the filter and remains in hemispherical drops during incubation. The relative numbers of migrated cells were determined using a MTT-assay.

**MTT assay.** For the quantitative determination of migrated cells an MTT assay (Promega, Madison, WI, USA) was used, which is based on the cleavage of the yellow tetrazolium salt MTT into purple formazan by metabolically active cells. The solubilized formazan product can be photometrically quantified using an ELISA reader. An increase in the number of living cells results in an increase of total metabolic activity which leads to a stronger colour formation. The experiments were performed at least three times for each data point.

**Protein analysis.** CRP was obtained from two different companies (Sigma, Munich, Germany and Calbiochem, Basel, Switzerland) and analyzed in regard to the content of monomeric and pentameric CRP fractions. Native PAGE (polyacrylamide gel electrophoresis) was used to separate the mono- and pentameric fractions of the CRP. Aliquots of the CRP (20 µg) were run on a 10% acrylamide gel which was subsequently subjected to Coomassie blue staining. Destaining of the gel was achieved using a washing buffer containing 30% methanol and 10% acetate.

**Detection of CRP mRNA.** Real-time PCR (TaqMan® PCR) for CRP mRNA detection in the different HNSCC lines was performed with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Darmstadt, Germany) following the recently published protocol (8). In brief, CRP mRNA was detected in a multiplex real-time PCR reaction amplifying CRP and hypoxanthine phosphoribosyltransferase (HPRT) cDNA within the same tube. The HPRT co-amplification was used for normalization of the CRP amplification signal avoiding tube-to-tube variations and pipetting errors. All determinations were done in duplicate taking the mean of both reactions for quantitative analysis. Hep3B cDNA and water as a negative control that was amplified in each experiment in duplicate were used for internal quality control. The hepatoma-derived cell line Hep3B exhibits an inducible CRP transcription and has been studied extensively regarding its CRP expression. The same calibrator sample was used throughout the whole study. Forty-five cycles of amplification were performed at 62°C for 1 min and at 95°C for 15 sec. The level of significance was set at 50% of the maximum intensity of the dyes FAM (HPRT) and VIC (CRP/HEP3), respectively.

**Results**

**Secretion of CRP by permanent cell lines of HNSCC.** All the cell lines (BHY, PCI1 and PCI13) secreted between 0.3 and 0.7 µg/ml CRP. In addition, qualitative real-time PCR showed the presence of CRP mRNA in the different HNSCC cell lines. The internal control HPRT (22 samples) reached the level of significance after 19.97 cycles (s.d. 0.75). The internal control HEP3 (2 samples) reached the level of significance after 25.83 cycles (s.d. 0.08). The target CRP (11 samples) reached the level of significance after 40.49 cycles (s.d. 1.68). In the negative control neither HPRT nor CRP was detected. Low CRP mRNA levels were demonstrated in all the analyzed cell lines thus corroborating the detected secretion of C-reactive protein by these cells.

**Serum-CRP-levels of HNSCC patients.** The charts of 150 patients with HNSCC (laryngeal and pharyngeal cancer) were reviewed. The serum-CRP-levels of HNSCC-patients before therapy were 0-230 µg/ml with a mean value of 20 µg/ml. There was no correlation to the T-stage or grading.
Some low-grade-T4-cancer patients had a normal serum CRP-level, while some T1-cancer patients had serum-CRP-levels up to 200 μg/ml.

Pentameric CRP effect on the migration of human MDC. Marked differences of approximately five fold in the proportions of mono- and pentameric CRP were found between the Sigma and Calbiochem CRP samples (Figure 1).

Cell migration following incubation of MDC with each type of CRP is shown in Figure 2. At a concentration of 10 μg/ml Calbiochem CRP the maximum decrease of MDC migration was demonstrated, whereas 100 μg/ml of Sigma CRP were required to achieve a comparable down-regulation. These data clearly indicated that pentameric CRP was the biologically active and critical fraction.

CRP effect on chemokine receptor CCR5 on human MDC. Flow cytometry was used to analyze the surface expression of CCR5 on human MDC in response to increasing CRP concentrations. Decreasing surface expression levels of CCR5 were shown in response to increasing concentrations of CRP (Figure 3). These data confirmed the observed decrease of MDC cellular migration in response to the MIP-1β chemoattraction.

Discussion

Human solid tumor tissues of HNSCC are known to be infiltrated by various kinds of immune cells, but the immunosuppressive HNSCC microenvironment leads to substantially impaired immune responses (13-15). Alterations in immune, inflammatory and angiogenetic responses within the HNSCC microenvironment have been reported to play a critical role in tumor aggressiveness and its influence on the cellular immune system.

Recent data have suggested a partial Th2 cytokine bias within the HNSCC microenvironment, as well as an aberrant expression of Th2 cytokines such as IL-4, IL-6 and IL-10 in the plasma of patients with a more advanced disease (5, 20, 22). MIP-1β is known to be the ligand for chemokine receptor CCR5 which is expressed by various immune cells such as monocytes, dendritic cells and Th1 cells, and has been involved in memory responses (23, 24).

In renal cell carcinoma (RCC), rises in plasma CRP have been reported to indicate tumor progression (12, 25, 26). Our data showed the first evidence of autonomous production of the acute-phase protein CRP by cells of head and neck squamous cell carcinoma. In one study the immunohistochemical production of CRP by squamous cell carcinoma of the esophagus was described; however this observation was not confirmed by transcriptional analysis of the CRP gene within the tumors (27). The authors concluded that intratumor CRP expression along with high plasma CRP levels identified patients with unfavorable outcomes. Another group showed an inducible expression of CRP by the human lung epithelial carcinoma cell line A549 (28), which again suggested that CRP expression by malignant cells is not restricted to RCC.
In the present study CRP had a marked influence on the migration activity of MDC. However, in our retrospective review of patients charts the serum CRP levels did not correlate with the grading or size of the tumor. Patients with small as well as large tumors had serum CRP levels within the normal range or highly elevated levels. The tumor cells produced relatively small amounts of CRP as shown in the supernatants of the cell lines and suggested by mRNA amplification. It is unlikely that these small amounts lead to a high elevation of serum CRP levels. Pharyngeal and laryngeal tumors especially the ones with ulcerative growth are known to carry a bacterial superinfection. This is more likely to be the cause of highly elevated serum CRP-levels in HNSCC patients. To clearly identify a prognostic value of serum CRP levels in HNSCC patients a prospective study which also monitors the superinfection should be carried out.

Tumor cell derived CRP is able to influence the surface expression of molecules and migration activity of human MDCs. MDCs are characterized by their expression of surface proteins CD11c, HLA-DR and CD1c (human blood dendritic cell antigen-1, BDCA-1) as well as their lack of lineage markers (CD3, CD16, CD19, CD20, CD56).

Assuming that the serum level is not affected by this autonomous production the biological meaning should be investigated within the tumor environment. Therefore, further investigations concerning the physiological scale and function of CRP in vivo should be carried out with respect to its influence on cellular functions of the human immune system.

In summary permanent cell lines of HNSCC secrete notable levels of CRP, which were also found in the serum of patients with HNSCC. Increased concentrations of CRP result in a down-regulation of chemokine receptor CCR5 on human MDCs and a decreased migration activity of these cells.

Thus tumor derived CRP is a potential modulator of proper immune cell migration in patients with head and neck cancer. The concentration of the pentameric fraction of the C-reactive protein appeared to be a critical parameter of its immunomodulatory capacity.

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References


Figure 3. Decreased CCR5 expression in response to CRP. Flow cytometric analysis of surface CCR5 in human myeloid dendritic cells in response to incubation with C-reactive protein compared to the medium control.

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