Head and Neck Cancer Triggers Increased IL-6 Production of CD34+ Stem Cells from Human Cord Blood

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Abstract. Background: Head and neck squamous cell carcinoma (HNSCC) are infiltrated by various kinds of immune cells, which show massively impaired immune functions. The influence of HNSCC on CD34+ progenitor cells from human cord blood was analyzed. Materials and Methods: CD34+ cells were isolated from human cord blood by magnetic bead separation using magnetically labelled antibodies. Immunofluorescent staining of CD34+ cells in solid HNSCC was carried out. Cytokine levels of IL-6, IL-8, and IL-10 were analyzed with flow cytometry using the BD CBA Human Soluble Protein Flex Set system (Becton Dickinson). Results: We demonstrated that HNSCC triggered CD34+ cells to produce increased levels of the tumor-promoting cytokine IL-6 and thus they participate in the development of the microenvironment of head and neck cancer. Conclusion: HNSCC modulates the cytokine secretion profile of tumor infiltrating cells to escape from efficient immune responses and to trigger its own malignant progression.

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common tumor occurring almost exclusively among middle-aged tobacco and alcohol abusers (1). Cells of head and neck cancer are known to develop molecular strategies to escape from efficient anti-tumor immune responses. Human solid tumor tissues have been demonstrated to be infiltrated by various kinds of immune cells and it is supposed that tumor production of different immunomodulatory mediators contributes to massively impaired immune functions (2-4). The malignant transformation process is strongly associated with an altered response to cytokine stimulation which plays a critical role in tumor aggressiveness and its response to chemo- and radiation therapies, whereas the responsible molecular mechanisms remain mostly unknown (5-9). Prominent HNSCC cytokines are interleukin-4 (IL-4), IL-6, IL-8 and IL-10, granulocyte macrophage-colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), prostaglandin E2 (PGE2) as well as basic fibroblast growth factor (bFGF) (8-12).

It was shown for HNSCC that the mobilization of CD34+ natural suppressor cells in the bone marrow is stimulated via GM-CSF and that VEGF subsequently triggers their chemoattraction into the tumor (13, 14). These CD34+ progenitor cells are able to restrict the function of HNSCC infiltrating T-cells (15), whereas their suppressive character has been suggested to act through the production of nitric oxide (NO), as well as transforming growth factor (TGF)-β (16, 17).

Our data demonstrate that solid HNSCC is infiltrated by CD34+ stem cells. Traditionally, IL-6 is known to stimulate the liver to produce acute phase proteins or to trigger the proliferation of B-lymphocytes. But IL-6 was furthermore shown to possess pro- as well as anti-inflammatory properties and thus dysregulations of IL-6 cytokine signalling often contribute to various kinds of cancer (18, 19). IL-10 is a strongly immunosuppressive cytokine and involved in the down-regulation of interferon-γ (IFN-γ) and Th1 immune responses (20). In this work we analyzed the influence of HNSCC on CD34+ progenitor cells from human cord blood with respect to the secretion levels of cytokines IL-6, IL-8, and IL-10.

Materials and Methods

Cell culture. Permanent HNSCC cell lines BHY (DSMZ Germany) (21) and PCI-1 (Hypopharyngeal cancer, Pittsburgh Cancer Institute, PA, USA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, New York, USA) supplemented with 10% FCS, 1 mM glutamine, and 0.1 mM sodium pyruvate. All compounds were purchased endotoxin tested. Cell-free supernatants of these cell lines were prepared by several centrifugation steps.

Cytokine analysis. Cytokines IL-6, IL-8, and IL-10 were detected using the BD CBA Human Soluble Protein Flex Set system (Becton Dickinson, Heidelberg, Germany). This system uses the sensitivity of amplified fluorescence detection with flow cytometry to measure a soluble analyte. A BD™ CBA Human Soluble Protein
Isolation of CD34+ cells. CD34+ cells were isolated from human cord blood provided by the University Hospital of Lübeck. Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque density gradient centrifugation as described elsewhere (22). CD34+ cells were isolated via magnetic bead separation using magnetically labelled anti-CD34 antibodies (Miltenyi, Bergisch Gladbach, Germany).

Flow cytometry. Isolated cells were analyzed with flow cytometry using FSC and SSC properties and identified as a population of CD34-positive cells. Surface antigen staining was performed as described elsewhere (23). Cells were stained with fluorescein-5-isothiocyanate (FITC-) conjugated anti-CD34 antibodies and adenomatous-polyposis-coli (APC-) conjugated anti-CD38 antibodies (Becton Dickinson Heidelberg, Germany) by incubation on ice for 15 min followed by washing with PBS. Samples were analyzed on a FACSCanto (Becton Dickinson), Propidium-iodide staining was used to determine the number of dead cells. Data acquisition and analysis were performed using the FACS DIVA software (Becton Dickinson).

Immunofluorescence microscopy. For immunofluorescence staining, tissue specimens were embedded in Tissue Tek (Sakura Finetek, Torrance, CA, USA), cryopreserved in N₂ and stored at −20°C. Acetone-fixed cryosections (4-μm) were incubated with the FITC-conjugated anti-CD34 antibodies for 1 h at room temperature. After several washing steps, fluorescence microscopy was used to analyze the presence of CD34-positive cells in solid HNSCC. Samples were viewed on a Zeiss Axiosvert 200M microscope by either differential interference contrast (DIC) microscopy or fluorescence microscopy using a GFP filter set (AHF Analysentechnik AG, Tübingen, Germany). Samples were photographed using an Zeiss AxioCam MRm Rev.3 FireWire (D) and the Zeiss AxioVision Rel. 4.5 software.

Results

Isolation of CD34+ cells. CD34+ progenitor cells from cord blood were isolated by magnetic bead separation and analyzed by flow cytometry. The expression of the characteristic surface antigen CD34 was investigated. Magnetic bead separation of CD34+ cells resulted in average cell amounts of about 10^5 cells per ml of human cord blood. Flow cytometric analysis revealed average cell purities of enriched CD34+ cells between 90 and 95% (Figure 1). Flow cytometric analysis revealed no significant expression of lineage markers (CD3, CD14, CD16, CD19, CD20, CD56) in the isolated CD34+ cells from human cord blood (Figure 1).

Localization of CD34 expressing cells in solid HNSCC. CD34 staining of frozen sections of solid HNSCC clearly revealed the infiltration of CD34 expressing progenitor cells in these tissues. While CD34 in tissues is expressed not only by CD34+ progenitor cells but also by cells of blood vessels, staining with the FITC-conjugated anti-CD34 antibody demonstrated an increased localization of CD34+ cells next to tumor draining vessels (Figure 2).

Influence of HNSCC on CD34+ cells. We sought to investigate the influence of HNSCC on the activation and cytokine secretion of isolated CD34+ cells. Therefore, cells were incubated with cell-free supernatants of two different HNSCC cell lines (BHY, PCI 1) as well as with HNSCC cells of these two cell lines for 8 days at 37°C. Shorter time frames of incubation showed no significant effects.

Flow cytometric analyses of the characteristic activation marker CD38 revealed an increased activation of CD34+ cells in response to the incubation with supernatants and cells of HNSCC. Our data demonstrate a significantly increased expression of surface CD38, whereas HNSCC cells revealed a higher activating capacity compared to cell-free HNSCC supernatants (Figure 3).

We analyzed the secretion levels of cytokines IL-6, IL-8, and IL-10 by CD34+ cells in response to the influence of HNSCC. Our data show that the HNSCC microenvironment triggered an increased production of IL-6 by CD34+ cells. No significant effects on the IL-8 and IL-10 levels were observed (Figure 4).

Discussion

Appropriate immune responses are regulated at distinct molecular levels such as the manner of antigen presentation, the amount of antigen, individual genetic predispositions and/or the surrounding environment. The HNSCC microenvironment is composed of numerous immunosuppressive and immunomodulatory mediators which contribute to fatal immune dysfunctions (5).

Recent data suggested a partial Th2 cytokine bias in HNSCC patients which revealed increased levels of Th2 cytokines IL-4, IL-6, IL-10 and GM-CSF (19, 24, 25). It has also been shown that tumor infiltrating T-cells were skewed toward a Th2 cytokine profile (26). Elevated levels of cytokines IL-6 and IL-8 could furthermore be detected in the serum of patients with HNSCC, but not in those of patients with benign squamous papilloma (10). In cells of oral squamous carcinoma, the constitutive expression of cytokines IL-6 and GM-CSF has been found to be responsible for a down-regulation of the co-stimulatory molecule CD80 (19).

The precise role of various cytokines in HNSCC tumorigenesis and maintenance is yet unclear. Our work provides an additional novel piece of information concerning the origin of the HNSCC microenvironment and the role of CD34+ progenitor cells within this process. Since stem cells are extremely versatile with almost inexhaustible abilities of...
differentiation, it is supposed that they are involved in a huge variety of oncogenic processes such as immunomodulation, angiogenesis or metastasis. It has been demonstrated that tumor-derived cytokines led to an increased number of myeloid progenitor cells in animal models as well as in cancer patients (27, 28). In models of neovascularization, it has been shown that fluorescent-tagged human CD34+ progenitor cells became incorporated as endothelial cells into newly growing vessels (29).

Most of these functions and the responsible molecular mechanisms still have to be further elucidated. The understanding of the cytokine regulatory routes in HNSCC is a fundamental step for the development of novel immunotherapeutic strategies against HNSCC.

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References


Figure 3. Activation of CD34+ cells through HNSCC. Flow cytometric analyses revealed an increased expression of the characteristic activation marker CD38 in response to incubation with supernatants and cells of HNSCC.

Figure 4. HNSCC triggers an increased production of IL-6. Flow cytometric analyses of HNSCC relevant cytokines IL-6, IL-8, and IL-10 revealed an increased secretion of IL-6 in response to incubation with supernatants and cells of HNSCC.


