Polymorphonuclear granulocytes in human head and neck cancer: enhanced inflammatory activity, modulation by cancer cells and expansion in advanced disease

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The progression of epithelial cancer is associated with an intense immunological interaction between the tumor cells and immune cells of the host. However, little is known about the interaction between tumor cells and polymorphonuclear granulocytes (PMNs) in patients with head and neck squamous cell carcinoma (HNSCC). In our study, we investigated systemic PMN-related alterations in HNSCC, the role of tumor-infiltrating PMNs and their modulation by the tumor microenvironment.

We assessed the infiltration of HNSCC tissue by PMNs (retrospectively) and systemic PMN-related alterations in blood values (prospectively) in HNSCC patients (n = 99 and 114, respectively) and control subjects (n = 41). PMN recruitment, apoptosis and inflammatory activity were investigated in an in vitro system of peripheral blood PMNs and a human HNSCC cell line (FaDu). HNSCC tissue exhibited considerable infiltration by PMNs, and strong infiltration was associated with poorer survival in advanced disease. PMN count, neutrophil-to-lymphocyte ratio and serum concentrations of CXCL8 (interleukin-8), CCL4 (MIP-1b) and CCL5 (RANTES) were significantly higher in the peripheral blood of HNSCC patients than in that of controls. In vitro, HNSCC-conditioned medium inhibited apoptosis of PMNs, increased chemokinesis and chemotaxis of PMNs, induced release of lactoferrin and matrix metalloproteinase 9 by PMNs and enhanced the secretion of CCL4 by PMN. Our findings demonstrate alterations in PMN biology in HNSCC patients.

In vitro, tumor-derived factors modulate cellular functions of PMNs and increase their inflammatory activity. Thus, the interaction between HNSCC and PMNs may contribute to host-mediated changes in the tumor microenvironment.

Worldwide, head and neck cancer is one of the six most common cancers. More than 90% of head and neck cancers are squamous cell carcinomas (HNSCCs) that primarily originate in the oral cavity, the pharynx and the larynx.1–3 HNSCCs display an inflammatory microenvironment with frequent infiltration by large numbers of immune cells. This infiltration results in a reciprocal interaction between the malignant tissue and the immune cells that causes local and systemic alterations, often resulting in the downregulation of immune functions and the tumor escape from immune control.4–6 Accumulating evidence suggests that polymorphonuclear granulocytes (PMNs) and other myeloid cells play an important tumor-promoting role during tumor progression.7–9 High numbers of PMNs before treatment, as determined in the peripheral blood of patients with malignant melanoma,10 and an increased neutrophil-to-lymphocyte ratio (NLR), as demonstrated in ovarian cancer,11 have been proposed as independent prognostic factors for short overall survival. Tumor-infiltrating PMNs have been linked to a poorer prognosis for patients with lung adenocarcinoma of the bronchioloalveolar subtype,12 but they seem to be associated with a reduced mortality for patients with gastric carcinoma.13

PMN functions are modulated by a variety of cytokines and chemokines,14 and many of those factors have been implicated in tumor progression: CXCL8 (interleukin-8) promotes the tumor infiltration by PMNs15 and has been...
implicated in the modulation of the tumor microenvironment.\textsuperscript{16,17} In HNSCC patients, serum CXCL8 has been suggested as a possible biomarker for response and survival.\textsuperscript{18,19} CCL4 (macrophage inflammatory protein 1\textit{b}) is produced by a variety of cells including PMNs.\textsuperscript{20} It regulates the recruitment of both myeloid and lymphoid immune cells\textsuperscript{21} and their intratumoral infiltration.\textsuperscript{22} In contrast to some antitumor activity of CCL4,\textsuperscript{22} recent reports have linked an overexpression of CCL4 with tumor recurrence or progression.\textsuperscript{23,24} CCL5 (RANTES) promotes PMN chemotaxis,\textsuperscript{25} seems to be a marker of disease progression for breast cancer patients\textsuperscript{26} and has been shown to increase the production of matrix metalloproteinase 9 (MMP-9) by oral cancer cells.\textsuperscript{27}

It has been reported that tumor cells actively modulate the functions of PMNs. For example, the expression of CXCL8 by tumor cells promotes the recruitment of PMNs to the tumor and their activation.\textsuperscript{28} Recruited PMNs exhibit increased production of reactive oxygen species, NADPH oxidase and myeloperoxidase (MPO).\textsuperscript{29} In bronchialalveolar carcinoma, the local survival of PMNs is prolonged by the production of antiapoptotic factors by the tumor microenvironment.\textsuperscript{30}

In our study, we investigated local and systemic PMN-related alterations in patients with HNSCC. We used an \textit{in vitro} system to investigate the functional interaction between HNSCC cells and PMNs. We found that HNSCC cells upregulate inflammatory activity and also upregulate the production of factors in PMNs with the possible ability to promote tumor progression. HNSCC patients exhibit increased expression of the chemokines that regulate PMN biology. The intratumoral accumulation of PMNs was associated with poor survival in advanced disease.

\section*{Material and Methods}
\textbf{Study subjects and tumor characteristics}

The experiments were approved by the local ethics committee, and written informed consent was obtained before sample collection. Blood samples were prospectively collected from patients before oncologic therapy and from 41 healthy volunteers as controls. Altogether, 114 patients (median age, 63 years; range, 41–86 years) with HNSCC of the oral cavity, oropharynx, hypopharynx or larynx were enrolled from 2008 to 2009 (survival analysis not yet available). For characteristics of patients and tumors, see Table 1. All consenting patients were included in the study unless they had HNSCC in other locations, radiotherapy or chemotherapy within the past 5 years, synchronous carcinoma in another location or severe concomitant infectious disease. HNSCCs were staged according to the tumor-node-metastasis (TNM) system.\textsuperscript{1} For tissue analysis, we retrospectively analyzed paraffin-embedded sections collected from 99 patients (median age, 59 years; range, 36–87 years) with HNSCC. No restriction of selection was used except of localization (oropharynx or hypopharynx), availability (tissue and clinical data) and date of first diagnosis (between 1995 and 2001) (see Table 1). Our focus was on advanced disease (Stage III or IV). Retrospective analysis of clinical courses shows that surgery alone was performed in 9%, surgery combined with adjuvant radio(chemo)therapy in 22% and primary radio(chemo)therapy in 69% of the patients (followed by salvage surgery in 36% of these). The median follow-up period for surviving patients was 69 months (range, 43–124 months).

\section*{Peripheral blood analyses}

Peripheral blood measurements included differential hemogram, C-reactive protein (CRP) concentration, determined with automated analyzers (hemogram, Sysmex XE5000,
with Aqua Braun (B. Braun). The resulting PMNs (purity of (1:1 v/v) (Sigma-Aldrich). Remaining erythrocytes were lysed with 1% polyvinyl alcohol solution (1:1 v/v) with PBS and separated by gradient centrifugation. Erythrocytes were sedimented with 1% polyvinyl alcohol solution (1:1 v/v) with PBS and separated by gradient centrifugation.

**Immunohistochemistry**

Immunohistochemical staining for visualization of CD66b-positive cells was performed with an automated staining device (Dako REAL Peroxidase-Blocking solution (DakoCytoCytomation), citrate buffer pH 6.0 (Invitrogen) and TBS containing 1% bovine serum albumin (PAA). Samples were incubated overnight with monoclonal mouse anti-human MPO antibodies (AbD Serotec, MorphoSys) (1:1,000), followed by peroxidase-conjugated rabbit anti-mouse and anti-rabbit antibodies (1:50) (Dianova) and AEC Single Solution (Zymed Laboratories, Invitrogen). Nuclei were visualized by Shandon Instant Hematoxylin (Thermo Fisher Scientific). Sections were analyzed with a Zeiss Axioskop 2 (Zeiss) using objective lenses with 40× and 63× magnification and AxioVision software (Zeiss).

**Culture of HNSCC cell line**

In our study, we used the human hypopharyngeal carcinoma cell line FaDu (American Type Culture Collection, ATCC). FaDu cells were cultured in RPMI-1640 (Invitrogen) supplemented with 10% fetal calf serum and antibiotics (Biochrom). Quality and identity of the cell line were validated consistently according to ATCC Technical Bulletin No. 8, including regular microscopic controls of morphology, growth curve recordings and PCR-based testing for mycoplasma infection. FaDu-conditioned medium was produced by incubating 2 × 10^6 FaDu cells per milliliter for 24 hr at 37°C in RPMI-1640. Cellular debris was removed by centrifugation.

**Isolation of PMNs from peripheral blood**

We used previously established protocols for isolation of PMNs. Peripheral blood from healthy subjects was diluted (1:1 v/v) with PBS and separated by gradient centrifugation with 1077-Lymphocyte Separation Medium (PAA). Erythrocytes were sedimented with 1% polyvinyl alcohol solution (1:1 v/v) (Sigma-Aldrich). Remaining erythrocytes were lysed with Aqua Braun (B. Braun). The resulting PMNs (purity of >98%) were cultured in RPMI-1640 supplemented as above.

**Migration and apoptosis of PMNs**

Directed migration (chemotaxis) and random migration (chemokinesis) of PMNs were examined by using 3-μm cell culture inserts in 24-well companion plates (BD Bioscience). The companion plates were filled with 800 μl of medium with 5 ng/ml recombinant CXCL8 (R&D Systems) or FaDu supernatant, in the presence or absence of anti-CXCL8-neutralizing antibodies (R&D Systems) or isotype control (BD Pharmingen), respectively. PMNs (5 × 10^5 cells per 200 μl) were placed in the inserts, allowed to migrate for 3 hr at 37°C and migrated cells were counted (CASY Model TT; Innovatis). The migration/chemotactic index is calculated according to the following formula: chemotactic index = migration induced by the chemoattractant/spontaneous migration toward control medium. By definition, spontaneous migration of PMN in control medium has a chemotactic index of 1.

To measure apoptosis, we stained PMNs with PE-Annexin V and 7-amino-actinomycin D according to the manufacturer’s instructions (BD Pharmingen). Quantification was performed with a FACSCanto II flow cytometer (BD). We performed independent experiments using PMNs from n = 15 (for chemotaxis), n = 7 (for chemokinesis) and n = 3 (for apoptosis) donors.

**CCL4, lactoferrin and MMP-9 ELISA**

To measure CCL4 release, purified PMNs (1 × 10^6 cells per milliliter) were left untreated or stimulated with 10 ng/ml lipopolysaccharide (LPS) (as a surrogate model of inflammation; kindly provided by Prof. Dr. K. Brandenburg, Research Center Borstel, Borstel, Germany) during 24-hr incubation in medium or 1:4 diluted FaDu supernatant. CCL4 release by PMNs was determined by enzyme-linked immunosorbent assay (R&D Systems) with a Synergy 2 microplate reader (BioTek). To measure lactoferrin and MMP-9 release, purified PMNs were incubated in the presence or absence of FaDu-conditioned medium for 90 min. Lactoferrin (Abnova, Taipei, Taiwan) and MMP-9 (R&D Systems) concentration in the supernatant were assessed after 15, 45 and 90 min by ELISA.

**Statistical analysis**

Standard descriptive statistics were used (e.g., means and standard deviations). To assess between-group differences, we used nonparametric exact tests throughout (Mann–Whitney–Wilcoxon U-tests for two groups or Kruskal–Wallis tests for more than two groups). Correlation coefficients reported are (Spearman) rank correlations. Survival time in months was calculated as the difference between diagnosis date and date of death independent of the cause or last observation date in case of censoring. Although survival probabilities were graphically assessed by the Kaplan–Meier method, univariate and multivariate Cox regression analyses were used for inference both in the total tissue samples (n = 99) and in a more homogeneous subsample (n = 40) (see Results). For the in vitro experiments, we used two-sample Student’s t-tests.

All reported p values are nominal, two sided with an α significance level of 0.05 and not adjusted for the testing of
multiple hypotheses. SPSS Version 16 (SPSS) was used for statistical analyses.

Results

PMN-related alterations in the peripheral blood of HNSCC patients

In a first attempt to assess the role of PMNs in the biology of HNSCC, we analyzed differences related to blood count. Study subjects included 41 controls and 114 patients with HNSCC. Patient and tumor characteristics are reported in Table 1, whereas descriptive summaries of blood count–related markers are displayed in Table 2 (for male patients). Peripheral blood data of female patients (n = 15) are shown separately in Supporting Information Table S1 in consideration of the well-known gender-specific (patho)physiological differences in blood count and immune function. Despite those differences and the small cohort examined, similar tendencies of results were observed for females.

The percentages of PMNs and the leukocyte counts were higher in HNSCC patients than in control subjects (p < 0.001) (Table 2). Because the total lymphocyte counts were similar in HNSCC patients and in controls, this resulted in a significantly higher NLR in HNSCC patients than in controls (p < 0.001). Further analysis indicated that PMN numbers, leukocyte counts and NLR were associated with the T stage of the tumor (with significantly higher numbers in T4 stage) (see Table 2) and with the lymph node involvement (N) stage (PMNs, p = 0.048; leukocytes, p = 0.018; NLR, p = 0.027; data not shown). These results were supported by correlation testing using the tumor size as measured either by endoscopic examination or by radiologic imaging (data not shown). Taken together, these findings indicate that the increase of PMN count is the most important difference in

| Marker          | T category | Mean (SD) | Median | Range       | p*          |
|-----------------|------------|-----------|--------|-------------|-------------|
| Leukocytes (nl⁻¹) | Controls   | 5.76 (1.92) | 5.21   | 3.12, 9.93  | † < 0.001   |
|                 | T1–T4      | 8.80 (2.72) | 8.21   | 4.15, 18.44 | † = 0.007   |
| PMN (%)         | Controls   | 49.6 (6.5)  | 48.3   | 41.4, 63.4  | † < 0.001   |
|                 | T1–T4      | 67.1 (9.4)  | 68.5   | 46.4, 88.4  | † = 0.006   |
|                 | T1         | 63.5 (6.9)  | 64.6   | 51.3, 73.1  | ¥ = 0.055   |
|                 | T2         | 63.3 (10.1) | 68.8   | 46.4, 74.6  | || > 0.1    |
|                 | T3         | 66.1 (5.2)  | 66.7   | 57.4, 73.0  |             |
|                 | T4         | 71.0 (10.5) | 71.1   | 51.7, 88.4  |             |
| PMN (nl⁻¹)      | Controls   | 2.73 (1.13) | 2.49   | 1.35, 5.47  | † < 0.001   |
|                 | T1–T4      | 6.12 (2.74) | 5.46   | 2.67, 14.16 | † = 0.003   |
|                 | T1         | 5.13 (2.54) | 4.28   | 2.84, 10.28 | ¥ = 0.030   |
|                 | T2         | 5.16 (1.47) | 5.39   | 3.31, 8.04  | || = 0.048   |
|                 | T3         | 5.06 (1.04) | 4.75   | 3.90, 7.32  |             |
|                 | T4         | 7.54 (3.35) | 7.10   | 2.67, 14.16 |             |
| NLR             | Controls   | 1.43 (0.50) | 1.28   | 0.89, 2.82  | † < 0.001   |
|                 | T1–T4      | 3.97 (2.46) | 3.50   | 1.24, 12.82 | † < 0.001   |
|                 | T1         | 2.64 (0.83) | 2.56   | 1.32, 4.01  | ¥ = 0.003   |
|                 | T2         | 2.95 (1.15) | 3.42   | 1.24, 4.35  | || = 0.027   |
|                 | T3         | 3.21 (0.77) | 3.33   | 1.88, 4.74  |             |
|                 | T4         | 5.40 (3.18) | 4.38   | 1.29, 12.82 |             |
| CRP (mg/ml)     | Controls   | 0.45 (0.87) | 0.10   | 0, 3.60     | † < 0.001   |
|                 | T1–T4      | 1.99 (3.04) | 1.00   | 0, 19.10    | † = 0.001   |
|                 | T1         | 0.73 (0.86) | 0.40   | 0, 2.60     | ¥ = 0.004   |
|                 | T2         | 1.52 (2.68) | 0.50   | 0, 12.90    | || = 0.001   |
|                 | T3         | 1.26 (1.29) | 0.80   | 0, 4.50     |             |
|                 | T4         | 3.19 (4.05) | 1.85   | 0.1, 19.10  |             |
*For each variable, the first p value (†) was determined by Mann–Whitney U-test with the factor tumor diagnosis (controls versus T1–T4), the second (‡) with the factor T category (T1–T3 versus T4), the third (§) if shown by Kruskal–Wallis test also with the factor T category (T1, T2, T3 and T4) and the fourth (||) if shown with the factor lymph node involvement (N0–N3; raw data not shown). Abbreviations: CRP: C-reactive protein; HNSCC: head and neck squamous cell carcinoma; NLR: neutrophil-to-lymphocyte ratio; PMNs: polymorphonuclear granulocytes.
the leukocyte composition of peripheral blood obtained from healthy donors and HNSCC patients.

Next, we assessed the presence of inflammatory mediators in the peripheral blood. The serum concentrations of the chemokines CXCL8, CCL3, CCL4 and CCL5 in HNSCC patients and in control subjects are shown in Figure 1; the serum concentrations of CRP are shown in Table 2. We found that the serum concentrations of CXCL8, CCL4 and CCL5 were significantly higher in HNSCC patients than in controls. In contrast, there was no significant difference between the groups for CCL3. CRP concentrations were positively and significantly associated with the T stage \( p = 0.004 \) and the N stage of the tumor \( p = 0.001 \). In a subgroup of patients with tumors of the pharynx and the oral cavity, tumor size as determined by endoscopy (Spearman rank correlation), T stage (Kruskal–Wallis test) and presence of nodal metastasis (Mann–Whitney U-test) were positively and significantly associated with CXCL8 (tumor size, \( r = 0.44, p = 0.023 \); T stage, \( p = 0.018 \); N0 versus N1–N3, \( p = 0.017 \)). Except for N stage, such associations were also observed for CCL4 (tumor size, \( r = 0.51, p = 0.006 \); T stage, \( p = 0.049 \); N0 versus N1–N3, \( p = 0.006 \) (data not shown). Taken together, these findings indicate that inflammatory activity in the peripheral blood of HNSCC patients is enhanced and that this enhanced activity is positively correlated with disease stage.

**Increased tumor infiltration by PMNs in advanced disease**

Increased chemokine concentrations may result in further activation of immune effector cells and in the recruitment of these cells to the tumor site. To investigate whether HNSCC tissue is infiltrated by PMNs, we stained tissue from 99 HNSCC patients for the granulocyte marker CD66b. We found that 93% of the tissue samples exhibited PMN infiltration in tissue areas consisting primarily of carcinoma cells or in stromal tissue regions (Fig. 2a). We further characterized the HNSCC tissue–associated PMNs by using immunohistochemical staining against the azurophilic (primary) granule marker MPO. We found that HNSCC tissues were infiltrated by MPO-positive cells (Fig. 2b). However, we also found cells that were morphologically recognizable as PMNs but that did not stain positive for MPO. These results could indicate that some of the tumor-infiltrating PMNs may have already executed their phagosomal oxidative burst and activity, although experimental artifacts cannot be excluded. Upon scoring the extent of PMN infiltration by using anti-CD66b staining (Fig. 2a), we observed a weak infiltration in 44% of the tissues, a medium infiltration in 30% and a strong infiltration in 19% (data not shown). When we compared PMN infiltration with tumor stage, most of the T4 tumors displayed medium or strong infiltration, whereas smaller and less-invasive tumors exhibited a lower degree of PMN infiltration (Fig. 2c).

These findings demonstrate that a considerable number of HNSCC cancers are infiltrated by PMNs and suggest a functional interaction between HNSCC cells and PMNs. **Tumor infiltration by PMNs is associated with poor survival in advanced disease**

To assess the clinical and pathophysiological relevance of HNSCC-infiltrating PMNs, we analyzed the relationship between the extent of PMN infiltration and the clinical outcome. To eliminate variables associated with the difference in disease stage and general health condition, we included only patients with advanced disease (Stage III or IV) and excluded those aged 70 years or more, those with the appearance of synchronous or metachronous cancer and those with severe systemic disease (ASA ≥ 2) at the time of initial diagnosis. For this subgroup, the 5-year survival rate was 40% (Fig. 2d). Although analysis of the whole cohort \( n = 99 \) yielded no association between PMN infiltration and survival, univariate and multivariate Cox regression analyses of patients with advanced disease \( n = 40 \) demonstrated that the 5-year survival rate for patients with medium or strong PMN infiltration was significantly lower than that of patients with weak or no infiltration \( p = 0.045 \) and \( p = 0.048 \), respectively) (Fig. 2d and Supporting Information Table S2). These findings indicate that the strong infiltration of HNSCC tissue by PMNs may represent a negative prognostic factor for HNSCC patients with advanced disease.

![Figure 1. Increased inflammatory mediators in the peripheral blood of male HNSCC patients. Serum concentrations of CXCL8 (a), CCL3 (b), CCL4 (c) and CCL5 (d) were measured in HNSCC patients (n = 42) and healthy subjects (n = 17). Shown are the median, percentiles (10th, 25th, 75th and 90th) and mean (dashed line) as vertical boxes with error bars; outliers are shown as dots. p values (Mann–Whitney) are indicated. For female patients, see Supporting Information Table S1.](image-url)
Modulation of PMN functions by HNSCC cells

In our final series of experiments, we set up an in vitro system to investigate cell biological mechanisms of HNSCC–PMN interaction. To this end, peripheral blood PMNs were stimulated with supernatant obtained from a human HNSCC cell line (FaDu).

We initially investigated the effect of FaDu-HNSCC cells on the migration and recruitment of PMNs. To assess...
random migration (chemokinesis), PMNs were stimulated with FaDu-conditioned supernatant or with control medium and allowed to migrate toward control medium. To assess directed migration (chemotaxis), PMNs incubated in control medium were allowed to migrate toward FaDu-HNSCC-conditioned supernatant or control medium. Counting the migrated cells after 3 hr of incubation demonstrated that, compared to the control medium, the tumor supernatant induced PMNs to respond with higher random migration (threefold) and higher directed migration (fourfold) (Fig. 3a). To investigate the mechanism of PMN chemotaxis, we used neutralizing antibodies against CXCL8. CXCL8 is a chemoattractant, which we found in high amounts (around 1 ng/ml) in FaDu-conditioned supernatant, in contrast to CXCL1 and CXCL6, which were not detectable (data not shown). The results show that neutralizing CXCL8 reduced PMN chemotaxis toward FaDu supernatant (Fig. 3b). Control experiments with recombinant CXCL8 demonstrated the potency of the inhibitory antibody as it reduced CXCL8-induced PMN chemotaxis to background levels (chemotactic index of 1). Fluorescence immunohistochemistry confirmed considerable expression of CXCL8 also in tissue sections from HNSCC patients (Supporting Information Fig. 1).

Next, to determine the effect of HNSCC cells on PMN survival, PMNs were incubated with FaDu supernatant or control medium, and apoptosis was determined 8 and 24 hr later. The survival of PMNs was significantly higher in the presence of tumor cell supernatant compared to control medium: more than 80% of cells remained viable even after 24 hr of culture (Fig. 3c).

Upon activation, PMNs are known to release a multitude of inflammatory factors, such as cytokines or chemokines. We investigated the effect of the HNSCC cell line on the release of CCL4 by PMNs. We chose CCL4 because...
produce substantial amounts of CCL4 only after appropriate activation. Additionally, we have demonstrated that serum concentrations of this chemokine were elevated in HNSCC patients (Fig. 1). We observed that the tumor cell supernatant induced secretion of CCL4 by PMN to similar levels as LPS. In addition, tumor cell supernatant enhanced LPS-induced CCL4 release by PMN (Fig. 4a). Because activated PMNs can also release factors that are contained in their granules, we determined the effects of HNSCC cell line supernatant on the release of lactoferrin (a marker for secondary granules) and MMP-9 (a marker for tertiary granules). The results indicated that, already 15 min after stimulation with tumor supernatant, the levels of both lactoferrin and MMP-9 in the supernatant were induced. These results show that PMNs are activated and rapidly degranulate after exposure to HNSCC cells (Figs. 4b and 4c).

In sum, these findings demonstrate that FaDu-HNSCC cells can modulate important cellular responses of PMNs, such as migration, apoptosis and the release of inflammatory factors, all of which may ultimately have important consequences for the tumor microenvironment and for tumor-associated inflammation.

Discussion

Recent studies using murine tumor models or involving cancer patients have provided evidence for an important functional role of PMN during tumor progression.35,36 In our study, we investigated the modulation of granulocyte immunobiology in human HNSCC. Our findings suggest that the infiltration of HNSCC tissue by PMNs is associated positively with tumor stage and negatively with overall survival times. We also observed systemic differences in the PMN compartment in HNSCC patients, which correlated positively with tumor size. In vitro experiments demonstrated that HNSCC cells directly recruit PMNs, prolong their survival and promote their inflammatory activity. The findings of these in vitro experiments are supported by the finding that the serum concentrations of the inflammatory chemokines CCL4, CCL5 and CXCL8 are higher in the peripheral blood of HNSCC patients than in that of controls. Thus, our study indicates that PMNs are important mediators of tumor-associated inflammation and may influence the survival of HNSCC patients.

Solid tumors often show a high degree of leukocytic infiltration and a state of so-called cancer-related inflammation. As part of this inflammatory process, various leukocyte...
subsets are recruited to the malignant tissue, where they contribute to tumor progression. Although the roles of tumor-associated macrophages, regulatory T cells, tumor-infiltrating lymphocytes and, more recently, myeloid-derived suppressor cells (MDSCs) in tumor progression have been intensively investigated, the impact of PMNs is less clear. This fact is surprising, because PMNs are the most abundant cell type in the peripheral blood. They secrete many potential immunomodulators, activate various other immune cells and, thus, play a role in many inflammatory diseases. However, recent reports have provided strong evidence for an important role of PMNs in tumor-host interaction. In a murine model, two differing polarized populations of tumor-associated neutrophils (TANs) were characterized. Transforming growth factor-β within the tumor microenvironment induces a protumorigenic population of TANs, whereas its blockade results in the recruitment and activation of TANs that are switched to an antitumor phenotype. Indeed, the impact of PMNs on tumor growth is characterized by dichotomous effects: PMNs function as antitumor effector cells in therapeutic settings such as bacterial immunotherapy or anti-body-dependent cellular cytotoxicity. As tumor-promoting effector cells, infiltrating PMNs may, for example, contribute to tumor angiogenesis. Furthermore, activated PMNs seem to influence the course of cancer disease by their immunosuppressive effects and inhibition of T-cell functions. Recently, intratumoral CD66b-positive PMNs have been described as an independent negative prognostic factor for renal cell carcinoma patients.

In our study, we observed several alterations in HNSCC patients related to peripheral blood and tumor-infiltrating PMN. Most HNSCC tissues were infiltrated by PMNs, and the degree of infiltration was positively correlated with the local tumor stage. Additionally, HNSCC cells seem to be a crucial trigger for the recruitment of PMNs. Our in vitro experiments showed that the mobility and migration of PMNs toward an HNSCC-conditioned medium are higher than toward a control medium. PMNs exposed to a culture environment conditioned with HNSCC cells demonstrated prolonged survival and enhanced inflammatory activity. This increased PMN activity may result in both protumor and antitumor effects. In particular, we observed that the release of lactoferrin, CCL4 and MMP-9 is increased when PMNs are exposed to HNSCC-conditioned medium. Lactoferrin is an important component of secondary granules in PMNs and has primarily been associated with antitumor effects. Although the role of CCL4 during tumor development is controversial at present (see Introduction), MMP-9 produced by tumor-infiltrating PMNs may play a crucial role in activating tumor angiogenesis and may contribute to carcinogenesis and further tumor progression.

In addition to evaluating the direct and local interaction between HNSCC cells and PMNs, we also analyzed systemic differences related to PMNs and inflammation in our patient cohort. Elevated serum concentrations of factors used as markers of inflammation, such as CRP and amyloid A, may be associated with poorer long-term survival, as has recently been shown for breast cancer. Cytokine profiles including proinflammatory IL-6 are modulated in patients with advanced HNSCC. Furthermore, serum CXCL8, an important cytokine in PMN biology, is found in HNSCC cell lines, tissue as well as serum and has been suggested as a possible biomarker for response and survival in HNSCC patients. Our findings further support the relevance of inflammatory biomarkers in HNSCC. We found that the serum concentrations of CRP and CXCL8 are higher in cancer patients than in controls and correlated positively with the size of the tumor. Furthermore, we have shown that an HNSCC-conditioned culture environment promotes the secretion of CCL4 by PMNs. Both the serum concentration of CCL4 and the infiltration of tumor tissue by PMNs are associated positively with tumor stage. Taken together, these findings suggest that PMNs recruited by the tumor may be a source of inflammatory mediators in HNSCC and may influence disease progression. CCL4 released at the tumor site may further recruit mononuclear immune cells, such as T lymphocytes, natural killer and immature dendritic cells, thereby increasing the inflammatory tumor–host interaction.

A recent report documented the presence of activated CD66b-positive PMNs in the peripheral blood of renal cell carcinoma patients and related those PMNs to the so-called MDSCs. MDSCs are a heterogeneous, partly granulocytic population of myeloid cells found in the spleens and tumors of mice with cancer. In mice, they are believed to suppress antitumor immunity by mechanisms involving arginase 1, nitric oxide and reactive oxygen intermediates, among others. However, the functional relevance of those putative peripheral blood MDSCs to tumor progression in humans remains unclear at present. We have observed that HNSCC tissue is infiltrated by CD66b-positive PMNs and that human HNSCC cells prolong the survival of PMNs in vitro. Our finding that the degree to which PMNs infiltrate advanced HNSCC tissues is negatively associated with the survival times of these patients indicates that PMNs may play an important pathophysiological role in HNSCC. We also show that tumor-derived factors directly modulate the cellular functions of PMNs and increase their inflammatory activity. Thus, tumor-infiltrating PMNs may be essential contributors to the inflammatory tumor–host interaction, and consequently, HNSCC patients may benefit from direct or indirect targeting of the inflammatory functions of PMNs.

Acknowledgements

The authors thank their colleagues at the Department of Otorhinolaryngology, University Hospital Essen, for their help with the acquisition of blood samples, and Petra Altenhoff and Anja Peglow for excellent technical support. This study was supported in part by the Rudolf Bartling Stiftung (S.L.).
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