Granulocyte Colony-Stimulating Factor and Granulocyte-Macrophage Colony-Stimulating Factor Promote Malignant Growth of Cells from Head and Neck Squamous Cell Carcinomas In vivo

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Abstract

Granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are used to ameliorate cancer therapy-induced neutropenia and mucositis. Yet, first data in head and neck squamous cell carcinoma (HNSCC) indicate an impaired long-term prognosis on G-CSF treatment, and previous studies showed a contribution of both factors to the progression of human epithelial tumors. Therefore, we investigate the role of G-CSF and GM-CSF in progression of tumor cells from human HNSCC. Both factors stimulated proliferation and migration of tumor cell lines established from patient tumors expressing G-CSF and GM-CSF and/or their receptors. Blockade of G-CSF and GM-CSF inhibited tumor cell invasion in a three-dimensional organotypic culture model. The contribution of both factors to tumor malignancy was further confirmed in nude mouse transplants in vivo. Invasive and malignant growth yielding a similar tumor phenotype as the original patient tumor was exclusively observed in G-CSF- and GM-CSF-expressing tumors and was associated with enhanced and persistent angiogenesis and enhanced inflammatory cell recruitment. Although factor-negative tumors grew somewhat faster, they were characterized by lack of invasion, reduced and transient angiogenesis, and large necrotic areas. These data provide evidence for a progression-promoting effect of G-CSF and GM-CSF in human HNSCC and suggest further detailed evaluation of their use in the therapy of these tumors. (Cancer Res 2006; 66(16): 8026-36)

Introduction

Tumor development and progression is not exclusively driven by the accumulation of genetic changes in the cancer cells (1), but rather requires a permissive and supportive tumor microenvironment (2, 3), induced by tumor-derived stroma modulating growth factors. Granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) were previously shown to stimulate tumor cell growth and migration in vitro (4–7) and to promote tumor progression in vivo by autocrine stimulation of tumor cells (6, 8) as well as paracrine activation of a tumor-supportive stroma (6, 9). Although originally identified as hematopoietic growth factors (10), both factors are also produced by a variety of nonhematopoietic cells, including fibroblasts, endothelial cells, and keratinocytes (11, 12). They induce the proliferation and migration of endothelial cells, thereby contributing to angiogenesis (13, 14), and can promote keratinocyte proliferation (5, 15, 16), resulting in a stimulatory role on wound healing (17–19). In normal cells (e.g., keratinocytes), the expression of both factors is strictly regulated (20), requiring induction by appropriate stimuli, such as interleukin-1, tumor necrosis factor-α, or lipopolysaccharides (16, 21). In contrast, the constitutive expression of G-CSF and GM-CSF, frequently together with their respective receptors, is found in numerous solid tumors (3, 22), such as skin or head and neck squamous cell carcinoma (HNSCC; refs. 6, 8, 18, 23, 24), gliomas (4), and meningiomas (9). G-CSF and GM-CSF contribute to tumor growth and progression by autocrine stimulation of proliferation and migration in skin SCC and gliomas (4–6) and by enhancing the invasive capacity of human lung cancer cells in vitro (7). GM-CSF expression correlates with the metastatic capability of different murine tumors (25), and expression of a GM-CSF transgene in mouse skin caused enhanced susceptibility to chemically induced skin tumors (14). Additionally, G-CSF and GM-CSF also promote tumor progression in a paracrine manner by contributing to an activated tumor stroma with enhanced angiogenesis and enhanced inflammatory cell infiltrate (3, 6, 21). Taken together, these data suggest an important role of G-CSF and GM-CSF in tumor progression.

On the other hand, due to their role as recruitment, proliferation, and maturation factors for granulocytes and macrophages as well as their stimulatory effect on wound healing, G-CSF and GM-CSF are commonly used in cancer therapy to ameliorate mucositis and neutropenia and to accelerate wound healing (18, 19, 26). In the therapy of HNSCC, oropharyngeal mucositis is a painful, often dose-limiting side effect of radiotherapy and chemotherapy (27). G-CSF and GM-CSF decrease the incidence of mucositis and GM-CSF directly promotes wound healing of the mucosa (19, 27). In addition, G-CSF and GM-CSF are used to prevent potentially life-threatening febrile neutropenia (28). Nevertheless, the survival benefit for patients under adjuvant therapy with G-CSF and GM-CSF is a matter of controversial discussion. The beneficial effect on neutropenia and mucositis is shown in several clinical trials (29, 30). Altundag et al. even hypothesize that use of G-CSF may stimulate the growth of breast cancer stem cells sensitizing them to chemotherapy, thereby increasing overall patient survival (31).

Nevertheless, several clinical trials have not shown a significant effect of G-CSF and GM-CSF on overall survival or disease-free survival, which might be partially due to the small sample sizes...
(28, 29, 32). A large randomized trial in advanced HNSCC even identified adjuvant G-CSF treatment as a poor prognostic factor with reduced locoregional control (33). Recent work has shown that G-CSF receptor signaling enhances the invasiveness of human HNSCC cells in vitro (24). Additionally, GM-CSF induces immunosuppressive CD34+ progenitor cells that interfere in host antitumor competence (34), thereby contributing to the decreased immune responsiveness in HNSCC-bearing mice (35). Accordingly, the expression of vascular endothelial growth factor (VEGF) and platelet-derived growth factor-AB together with G-CSF and/or GM-CSF expression correlated with a significantly poorer patient prognosis in HNSCC (23).

These controversial findings concerning the effects of G-CSF and GM-CSF on tumor growth and progression and their use in cancer therapy of HNSCC warrant a careful investigation of their functional effects on human HNSCC cells. We used four HNSCC tumor cell lines that were established from actual patient tumor specimens (23) and expressed both G-CSF and GM-CSF receptors. Two cell lines additionally expressed both factors, whereas the other two expressed neither factor. We could show a functional contribution of G-CSF and GM-CSF to tumor progression by an autocrine stimulation of tumor cell proliferation, migration, and invasion in vitro and by enhancing tumor invasion and malignancy in vivo.

Materials and Methods

Cell lines. Tumor cell cultures were established from surgical specimens of HNSCC and characterized for their epithelial origin and growth factor production (23).

HNO97(+) and HNO199(+) secrete G-CSF and GM-CSF, whereas HNO136(−) and HNO206(−) secrete neither G-CSF nor GM-CSF. VEGF and basic fibroblast growth factor (bFGF) expression in these cell lines was independent experiments with two replicas each.

Cell culture conditions. Cells were cultured in DMEM (Cambrex, Apen, Germany)-10% fetal bovine serum (FBS), penicillin/streptomycin (100 units/100 µg/mL), 1% L-ascorbic acid, and 5% CO2 in a humidified incubator, passaged at a split ratio of 1:6 to 1:15, and routinely tested negative for Mycoplasma contamination as described (36).

Conditioned medium and ELISA. Cells (2.5 × 10^3/cm²) were seeded into 10-cm culture dishes in DMEM-10% FBS and shielded from serum-free medium (105 µL/cm²) after 24 hours. After 96 hours, conditioned medium was harvested and stored at −80°C; viable cells were counted (Casy 1, Tuebingen, Germany). ELISA for G-CSF and GM-CSF was done [R&D Systems, Wieschen, Germany; human G-CSF (hG-CSF); DCS50 and human GM-CSF (hGM-CSF); DG00] according to the manufacturer's instructions. Samples were tested in duplicate. Data are mean of at least two independent experiments.

Growth curves. Cells (3 × 10^3/cm²) were seeded into 12-well plates in 300 µL/cm² DMEM-10% FBS. Medium was changed and cells were counted in triplicate after 24 hours and afterward every 48 hours up to 7 days.

Cell proliferation assay. Cells (3 × 10^4) were seeded in six replicas on 96-well plates in DMEM-10% FBS and shifted to DMEM-10% FBS with 0, 50, 100, and 150 ng/mL G-CSF or GM-CSF after 24 hours. Forty-eight hours later, 10 µmol/L bromodeoxyuridine [BrdU; Cell Proliferation ELISA, BrdU (colorimetric), Roche, Penzberg, Germany] was added to the wells for 16 hours and proliferation was analyzed according to the manufacturer's instructions. Mean of control samples containing no growth factor or antibody was arbitrarily set to 1. Values are mean of three independent experiments (six replicas each).

Cell migration assay. HNO97(+) (2 × 10^4), HNO199(+) (1 × 10^4), and HNO206(−) (1 × 10^4) or HNO136(−) (2 × 10^4) cells per well) were seeded in six-well plates. Twenty-four hours after confluence, the monolayer was disrupted by scraping and the lesions were marked. Medium was shifted to DMEM-10% FBS [HNO97(+) and HNO206(−)] or 1% FBS [HNO136(−) and HNO199(+)] containing 1, 10, or 50 ng/mL of either G-CSF or GM-CSF or 2 µg/mL of neutralizing antibodies against G-CSF or GM-CSF or an irrelevant antibody (mouse IgG1, Sigma-Aldrich, Munich, Germany). As additional control, 1 ng/mL G-CSF or GM-CSF plus 2 µg/mL of the respective neutralizing antibody (i.e., factor and antibody in compensating amounts) was added. Cell migration was documented by microscopic photos at time 0 and after 6 hours [HNO199(+)], 12 hours [HNO97(−)], or 24 hours [HNO136(−) and HNO206(−)]. Migration area was determined using the AnalySIS software (Soft Imaging Systems, Muenster, Germany). Data are mean of at least three independent experiments with 10 replicas each.

RNA isolation and reverse transcription-PCR. RNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany) and reverse transcription was carried out using 5 µg RNA, the Omniscript RT kit, and the Taq RNA polymerase (Qiagen) as described (36) in a volume of 100 µL. PCR contained 9 µL of the reverse transcription reaction and 2.5 units Taq DNA polymerase employing optimized MgCl2 concentration and annealing temperatures. PCR primers were as follows: G-CSF 5′-CACAGTGCACCTGACACGTG-CAGG and 3′-TACTAGGCTCTGACCTCCCTAATG, G-CSF receptor 5′-CTTGGAGCTGAAACTACCG and 3′-GCCACACAGAGTCTTGTCG, GM-CSF 5′-TGGGCTCGGACATCTCTGACGA and 3′-ACAGTTGGGCTGAGTGATG, GM-CSF receptor-a 5′-CTCTTCTGACCTAGCA and 3′-ACATGGGT-TTCTGAGTCG, and GM-CSF receptor-b 5′-AAATACAGATCCCTTTCAG and 3′-TACACCCACTGCTGACCAGAT.

All primers spanned intron-exon splice sites. The identity of the PCR amplification products was confirmed by size, restriction digest, and/or sequencing.

Tumorigenicity assays in vitro: organotypic cocultures. Dermal equivalents prepared from native type 1 rat collagen (3.2 mg/mL) containing 2.5 × 10^5 normal human dermal fibroblasts, were poured into polycarbonate membrane filter inserts (Falcon no. 3501, Becton Dickinson; refs. 3, 6, 21). Tumor cells (1 × 10⁶) were plated on top. After 24 hours, cultures were raised to the air-medium interface by lowering the medium level [DMEM-10% FBS, 50 µg/mL L-ascorbic acid (Sigma, Munich, Germany)]. In blocking experiments, fresh medium containing 3 µg/mL antibody against G-CSF, GM-CSF, or both was added every second day. For 3 weeks, two cultures per week were taken out and processed for histology and cryostat sectioning. Data are representative of two independent experiments with two replicas each.

Tumorigenicity assays in vivo: s.c. injection tumors. Tumor formation was assayed by s.c. injection of 5 × 10^6 cells into the interscapular region of 4- to 6-week-old nude mice (Swiss nu/nu). Growth of the resulting tumors was monitored by measuring tumor size in two axes and calculating the tumor volume as described (37). Tumors reaching a size around 1 cm³ were taken out and processed for histology and cryostat sectioning.

Matrix-inserted surface transplanation assay. Kinetics of tumor invasion, angiogenesis, and stromal activation were analyzed in surface transplants of tumor cells on collagen gels. Tumor cells (2 × 10^5) were grown for 1 day on a type 1 rat collagen gel (2.4 mg/mL) mounted between two concentric Teflon rings (Renner, Dannstadt, Germany). Before transplantation onto the dorsal muscle fascia of 6-week-old nude mice, the chamber was covered with a silicon hat (38, 39). For 6 weeks, three transplants per week were dissected and processed for cryostat sectioning. Data are representative of two independent experiments with three replicas for every time point.

Indirect immunofluorescence. Cryosections (5-6 µm) were mounted on slides, air dried, and stored at −80°C or directly fixed for 10 minutes in acetone at −20°C, air dried, and washed in PBS+ (Serva, Heidelberg, Germany). Slides were blocked with 10% bovine serum albumin for 15 minutes, washed in PBS, and incubated for 90 minutes with the primary antibody at room temperature. Subsequently slides were washed, incubated with the fluorescent secondary antibody and Hoechst bisbenzimid for 1 hour, washed, and mounted.

Staining with mouse antibodies in mouse tissue was done using a Zenon labeling kit (Molecular Probes, Leiden, the Netherlands) according to the manufacturer's instructions.

Apoptotic cells were stained using the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) in situ cell death
detection kit, TMR red (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Primary antibodies were as follows: hG-CSF and hGM-CSF (Calbiochem, Darmstadt, Germany); pan-cytokertatin and α-smooth muscle actin (Progen, Heidelberg, Germany); pan-cytokeratin (DAKO, Glostrup, Denmark); lC4D5 pan-leukocytes (Cymbus Biotechnology, Chemicon, Temecula, CA); lC6D8 monocytes, macrophages (BMA, Melsungen, Switzerland); lC6D6acg neutrophil granulocytes and murine neutrophils (Serotec, Düsseldorf, Germany); Ki-67 (MIB-1) and murine pan-macrophages (BM8; Dianova, Hamburg, Germany); murine CD31/platelet/ endothelial cell adhesion molecule 1 (PharMingen, BD Biosciences, Heidelberg, Germany); mCD45 pan-leukocytes (Leinco Technologies, St. Louis, MO); and BrdUrd (Exalpha, Maynard, MA).

**Quantification.** Photos of at least three immunofluorescent stainings of three different animals for each time point were analyzed using the AnalySIS software.

**Statistical analysis.** A two-tailed Mann-Whitney test was done using GraphPad Prism version 4.0a (GraphPad, San Diego, CA). Ps < 0.05 were considered significant.

**Results**

The functional role of G-CSF and GM-CSF in HNSCC growth and progression was analyzed using four HNSCC cell lines established from surgical specimens and previously thoroughly characterized for their epithelial origin (23). All cell lines expressed the G-CSF and GM-CSF receptors (data not shown). Two of the cell lines, HNO97(+) and HNO199(+), secreted G-CSF and GM-CSF in biologically active amounts, and two cell lines, HNO136(−) and HNO206(−), lacked both factors (Table 1). G-CSF- and GM-CSF-expressing tumors formed lymph node metastases in the patients, whereas G-CSF- and GM-CSF-negative tumors did not (23). Proliferative capacity of the cell lines in vitro was independent of their G-CSF and GM-CSF secretion (Fig. 1A) as were expression of additional growth factors, such as VEGF and bFGF (23). Factor and receptor expression in the tumor cells was found to be identical to the expression in the original patient tissues (23). Data for one cell line are representative for both cell lines with the same factor/receptor profile unless stated otherwise.

**Autocrine stimulation of tumor cells by G-CSF and GM-CSF in vitro.** G-CSF and GM-CSF clearly stimulate proliferation and migration of HNSCC tumor cells in vitro. All four cell lines showed a dose-dependent stimulation of cell proliferation in response to G-CSF or GM-CSF [for factor-positive HNO199(+); Fig. 1A]. The stimulation of tumor cell migration was equally as significant but required a lower factor concentration [for factor-negative HNO136(−); Fig. 1B]. Inhibition of migration in factor-expressing cell lines HNO97(+) and HNO199(+) with 2 μg/ml neutralizing antibody against G-CSF or GM-CSF [for HNO199(+); Fig. 1B] clearly showed an autocrine effect. Neutralizing antibodies had no effect on the factor-negative cell lines HNO136(−) and HNO206(−), and control experiments with irrelevant antibody or with factors and antibodies applied together in compensating amounts showed no effect (data not shown).

**G-CSF and GM-CSF promote tumor cell invasion in three-dimensional cultures in vitro.** G-CSF and GM-CSF critically influence tumor cell invasion as determined by blocking both factors with neutralizing antibodies in our three-dimensional organotypic tumor model in vitro (40). Both cell lines negative for G-CSF and GM-CSF formed massive epithelia with large necrotic areas on top of the fibroblast-containing collagen gel, showing no invasion into the gel [for HNO136(−); Fig. 1C]. Both tumor cell lines expressing G-CSF and GM-CSF infiltrated the collagen gel similar to SCC invasion in vivo [for HNO97(+); Fig. 1C]. Blockade of G-CSF alone using 3 μg/mL neutralizing antibody had no significant effect (Fig. 1D). However, tumor cell invasion was clearly reduced by GM-CSF blockade and almost completely abrogated by blockade of both G-CSF and GM-CSF (Fig. 1C and D).

**Tumor growth of HNSCC cells in vitro.** S.c. injection of tumor cell lines into the nude mouse resulted in progressively enlarging tumors. Interestingly, immunofluorescence staining against cytokeratins, Ki-67 for proliferation, CD31 for endothelial cells, and inflammatory cell markers, such as CD45+ (leukocytes), CD66acd+ (neutrophil granulocytes), and human CD68+ and mouse BM-8 antibody (macrophages), showed a histologic appearance of the s.c. tumor that was highly similar to the patient tumors from which the cell lines were established [for Ki-67 and keratin staining of HNO136(−) and HNO199(+); Fig. 2A and B]. Additionally, the nude mouse tumors retained the G-CSF and GM-CSF expression profile (Fig. 2C and D) that was observed in vitro and was shown previously for the patient tumors in vivo (23). The maintenance of these characteristics suggested the nude mouse as a suitable model to investigate the role of G-CSF and GM-CSF on tumor growth.

**G-CSF and GM-CSF decrease tumor growth rate but enhance tumor invasion.** G-CSF and GM-CSF expression strikingly affected in vivo growth behavior of the tumor cells. S.c. injection of G-CSF- and GM-CSF-expressing tumor cells [HNO97(+) and HNO199(+)] led to progressive tumor growth after ~3 weeks (Fig. 3A), whereas injection of factor-negative cell lines [HNO136(−) and HNO206(−)] gave rise to very fast growing tumors reaching a size of ~1,000 mm³ after 3 weeks (Fig. 3B). This seemed to contradict the growth-and invasion-promoting effect of G-CSF and GM-CSF in vitro. Yet, detailed histologic analysis revealed that G-CSF and GM-CSF

<p>| Table 1. G-CSF and GM-CSF expression data |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| <strong>Cell line</strong> | <strong>ELISA (pg/mL/10⁶ cells)</strong> | <strong>PCR</strong> |</p>
<table>
<thead>
<tr>
<th>G-CSF</th>
<th>GM-CSF</th>
<th>G-CSF</th>
<th>GM-CSF</th>
<th>G-CSF receptor-α</th>
<th>GM-CSF receptor-β</th>
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<tr>
<td>HNO97</td>
<td>129</td>
<td>42</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HNO136</td>
<td>0</td>
<td>0</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<tr>
<td>HNO199</td>
<td>1,244</td>
<td>584</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>HNO206</td>
<td>0</td>
<td>0</td>
<td>−</td>
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NOTE: mRNA and protein expression of G-CSF, GM-CSF, and the respective receptors in HNSCC cell lines as determined by reverse transcription-PCR and ELISA.
indeed promote tumor invasion and thus malignancy in vivo. The fast-growing factor-negative tumors exhibited a well-defined tumor stroma border with a rim of vital tumor cells surrounding a large central necrosis (HNO136(−); Fig. 3C) and lacked any obvious sign of tumor invasion (Fig. 2A and B). In contrast, the “slower”-growing factor-expressing tumors showed an extensive invasion of tumor cells into the host stroma and of vascularized stromal strands into the tumor mass (Fig. 2A and B), resulting in a highly invasive and malignant HNSCC phenotype.

In addition, tumor cells were grafted as heterotransplants using the surface transplantation assay (39) to study the kinetics of tumor development and tumor stroma interaction. In this system, factor-negative tumor cell lines developed predominantly noninvasive tumors with large necrosis on top (Fig. 3C, ii, HNO136(−)), whereas factor-positive cell lines formed highly invasive SCC with tumor cells invading the collagen gel as early as 1 to 2 weeks after transplantation and penetrating the underlying host stroma after 2 to 3 weeks [Fig. 3C, iii, HNO199(+); see also Fig. 4B].

We analyzed tumor cell proliferation, apoptosis, and necrosis to determine a potential reason for the faster tumor growth of factor-negative cell lines: the very high proliferation rate observed in surface transplants of factor-negative cell lines at day 7 remained constant throughout the experiment. In contrast, in factor-expressing tumors, proliferation started at a low rate and increased continuously, paralleling the increase in blood vessels density, ultimately reaching ~60% of factor-negative cell lines (Fig. 3D). TUNEL staining resulted in strongly positive signals in the necrotic areas of factor-negative tumors but showed almost no signal for apoptosis in the vital tumor parts (data not shown). Factor-expressing tumors exhibited no necrotic areas and few apoptotic

Figure 1. Effect of G-CSF and GM-CSF on tumor cell proliferation, migration, and invasion in vitro. A, stimulation of cell proliferation of G-CSF- and GM-CSF-expressing HNO199(+) by addition of G-CSF and GM-CSF. Growth curves indicate that the proliferative capacity of the cell lines is independent of their G-CSF and GM-CSF secretion. B, stimulation of cell migration of factor-negative HNO136(−) by G-CSF and GM-CSF and inhibition of cell migration of factor-expressing HNO199(+) by neutralizing antibodies to G-CSF and GM-CSF. C, H&E staining of HNSCC cells in a three-dimensional organotypic coculture after 21 days. Factor-negative tumor cells [HNO136(−)] show no invasion but extensive necrotic areas in the tumor epithelium (N, necrosis). Tumor cells expressing G-CSF and GM-CSF [HNO97(+)] exhibit extensive invasion reaching deep into the collagen gel. GM-CSF-neutralizing antibody (3 μg/mL) clearly reduced invasion and antibodies (3 μg/mL) against both G-CSF and GM-CSF together almost completely prevented invasion of G-CSF- and GM-CSF-expressing tumor cells [HNO97(+)]. Bar, 100 μm. D, quantification of invasion of factor-expressing HNO97(+) tumor cells in organotypic cultures in the absence or presence of G-CSF- and GM-CSF-neutralizing antibodies. *, P < 0.05.
signals in keratinized regions (data not shown). The 6-fold increase of TUNEL-positive staining in s.c. tumors of factor-negative compared with factor-expressing tumors was most likely due to the positive staining in necrotic areas (Fig. 3D). The constantly high tumor cell proliferation might be one reason for the fast increase in tumor size of the factor-negative cell lines, but the large necrotic areas indicate lack of vascularization and nutrition.

**G-CSF and GM-CSF contribute to tumor angiogenesis.**

To determine the influence of G-CSF and GM-CSF on angiogenesis and vessel maturation marked by pericyte coverage, tumor vessels were stained against CD31 (endothelial cells) and α-smooth muscle actin (pericytes). Overall, vessel density in s.c. tumors of factor-negative cell lines was reduced by ~40% compared with factor-expressing tumors (data not shown), whereas α-smooth muscle actin–positive mature blood vessels were increased 3-fold (Fig. 4A).

This difference also extended to the kinetics of angiogenesis and blood vessel maturation as shown in surface transplants. In surface transplants of G-CSF- and GM-CSF-expressing tumors, angiogenesis initiated as early as week 1 and was persistent. A continuously increasing number of vessels penetrated into the tumor mass during weeks 2 to 3, resulting in a highly vascularized tumor tissue (Fig. 4B and D). During the entire observation period, blood vessels associated with pericytes were rare (data not shown). Angiogenesis in surface transplants of factor-negative cell lines was significantly delayed. Blood vessels penetrated into the collagen gel in weeks 2 to 3 and reached the tumor-stroma border in week 3 (Fig. 4C and D). Infiltration of vessels into the tumor mass was observed only very rarely at the end of the observation period (6 weeks).

In contrast to the persistent angiogenesis of factor-expressing cell lines, angiogenesis was down-regulated at late time points of factor-negative tumors (Fig. 4D). The reduction in mean vessel density coincided with enlarged vessel diameters (Fig. 4C) and an increasing association of α-smooth muscle actin–positive cells with these enlarging vessels, suggesting progressive vessel maturation. Thus, expression of G-CSF and GM-CSF is linked with an early onset of an enhanced and persistent angiogenesis and a strong reciprocal invasion of tumor cells into the host stroma and stromal strands into the tumor mass.

**G-CSF and GM-CSF induce an earlier and enhanced inflammatory cell recruitment.**

G-CSF and GM-CSF regulate recruitment and differentiation of neutrophil granulocytes and macrophages that contribute to tumor invasion and angiogenesis by secreting angiogenic factors and matrix metalloproteinases (MMP; ref. 41). A significant effect of G-CSF and GM-CSF on neutrophil but also on macrophage recruitment was obvious in surface transplants of factor-expressing or factor-negative tumors. In the G-CSF- and GM-CSF-expressing tumors, recruitment of neutrophils was persistent, being prominent in the tumor stroma after 1 week and increasing continuously thereafter, reaching a 23-fold higher number than in factor-negative tumors. Neutrophils increasingly invaded the tumor mass [for HNO97(+); Fig. 5A and C]. The persistent neutrophil recruitment preceded the onset of angiogenesis in the factor-expressing tumors. In contrast, the recruitment of neutrophils into the tumor stroma of factor-negative tumors started only in week 2, reaching a plateau after 2 to 3 weeks and decreasing again in weeks 4 to 6 (Fig. 5B and C) following a similar kinetics as the transient angiogenesis. Neutrophil number in the tumor mass of factor-negative cell lines was always very low (Fig. 5C).

Additionally, macrophages recruitment to the stroma of G-CSF- and GM-CSF-expressing tumors was earlier with high macrophage numbers in the collagen gel already after 1 week, reaching a plateau in week 2. Macrophages invaded the tumor mass already in week 1 and increased dramatically in number after 3 weeks, reaching levels three times higher than in factor-negative tumors (Fig. 6A and C). The stroma of factor-negative tumors showed only 50% to 70% of the macrophage number compared with factor-expressing cell lines. Macrophage invasion into the tumor mass was delayed by 1 week and remained low (Fig. 6B and D). Taken together, expression of G-CSF and GM-CSF correlates with an accelerated recruitment of macrophages into the tumor neighboring stroma and
a dramatic increase of macrophage invasion into the tumor mass. This is accompanied by an enhanced and persistent recruitment of neutrophils and a strong increase of neutrophil invasion into the tumor mass. Neutrophil recruitment follows a similar kinetics as angiogenesis, being persistent in G-CSF- and GM-CSF-expressing tumors, whereas it is transient in factor-negative tumors. Together, persistent inflammatory cell recruitment and angiogenesis then contribute to the establishment of an invasive tumor phenotype.

Discussion

A stimulatory role of G-CSF and GM-CSF on tumor cells in vitro (4–7) and on tumor growth and progression in vivo (6, 8, 9) has recently been described for several tumor types. Yet, both factors are used in adjuvant tumor therapies to avoid adverse effects of radiotherapy and chemotherapy, such as mucositis and neutropenia (19, 26–28). This has raised questions not only with respect to their clinical benefit in adjuvant therapy but also with respect to their role in promoting tumor progression for clinically relevant tumor entities. Our data on the role of G-CSF and GM-CSF in tumor growth and progression of HNSCC, a tumor type where both factors are used in adjuvant therapy, show a stimulation of tumor cell proliferation and migration in vitro. Additionally, G-CSF and GM-CSF stimulate tumor invasion in an in vivo-like organotypic coculture. This can be blocked by antibody-mediated neutralization of GM-CSF and more so by blockade of both factors together. Having established these effects of G-CSF and GM-CSF on tumor cell proliferation, migration, and invasion in vitro, we investigated the in vivo relevance of these observations by s.c. injection and grafting of the tumor cells in our matrix-inserted surface
transplantation assay (39). The highly similar histologic phenotype of s.c. tumors and respective patients’ tumors, regarding their G-CSF and GM-CSF expression and their invasive growth behavior, angiogenesis, and cellular composition of the tumor tissue, suggested the nude mouse model as a valid tool to investigate the contribution of G-CSF and GM-CSF to HNSCC progression. Indeed, G-CSF and GM-CSF contribute to tumor progression through stromal activation in vivo by (a) promoting tumor invasion, (b) stimulating angiogenesis, and (c) enhancing the recruitment of inflammatory cells.

(a) The initial observation of faster s.c. tumor growth in factor-negative versus factor-positive cell lines seemed to contradict the progression-promoting effect of G-CSF and GM-CSF observed in vitro. However, histologic analysis revealed a more comprehensive picture: the higher tumor growth rate of factor-negative cell lines was most likely caused by their dramatically higher initial proliferation rate that remained constant throughout the experiment. Whereas in factor-expressing tumors necrosis was rare and the level of apoptotic cells was very low, factor-negative tumors showed extensive necrotic areas most likely caused by very fast tumor expansion and lack of sufficient tumor vascularization. Thus, their fast increase in tumor size was caused not only by a higher mass of vital cells but also by continuous enlargement of necrotic tumor areas, thereby mimicking a more aggressive tumor expansion. However, the most striking difference between factor-expressing and factor-negative tumors is tumor invasion: in factor-negative tumors, invasion was extremely rare. In contrast, invasion of factor-expressing cell lines was prominent in the s.c. tumors and

Figure 4. Kinetics of tumor angiogenesis in surface transplants of HNSCC tumor cells. A, quantification of α-smooth muscle actin–positive blood vessels in s.c. tumors of G-CSF- and GM-CSF-expressing [HNO97(+) and HNO199(+)] and factor-negative [HNO136(−) and HNO206(−)] tumor cells as percent tissue area exhibiting a 3-fold higher number of α-smooth muscle actin–positive vessel in factor-negative cell lines. Values were found to be significant for each factor-positive cell line compared with each factor-negative cell line. Immunofluorescent staining for keratin (green), CD31+ blood vessels (red), and nuclei (blue) of cryosections from surface transplants (14, 28, and 42 days) of G-CSF- and GM-CSF-expressing HNO199(+) (B) and factor-negative HNO136(−) (C) cells. Factor-expressing surface transplants show a persistent angiogenesis with extensive tumor invasion, whereas angiogenesis was transient followed by vessel enlargement in factor-negative surface transplants that lack tumor invasion. Bar, 200 μm. D, quantification of blood vessels in the stromal and tumor area of HNSCC tumors. In factor-expressing cell lines, angiogenesis initiated in week 1 in the stroma and was consistently increasing with vessels in the tumor mass after 2 weeks, whereas angiogenesis in factor-negative cell lines was delayed and weak. *, P < 0.05.
surface transplants with a strong reciprocal infiltration of tumor and stroma. Moreover, factor expression correlated with lymph node metastasis of the clinical tumors [HNO97(+) T3N2 and HNO199(+) T2N2], whereas factor-negative tumors were lymph node metastasis negative [HNO136(−) T4N0 and HNO206(−) T2N0; ref. 23]. Despite the enhanced proliferation in factor-negative tumors, it is the factor-positive cell lines that form the malignant, invasive, and metastasizing tumors, presenting the typical phenotype of highly malignant HNSCC. Although proliferation rate is quoted as a marker of malignancy in many human tumors, it is not always an adequate marker for malignant progression as shown for ovarian serous tumors, mammary tumors (42, 43), and tumors of the oral cavity (44, 45). The invasion and progression-promoting effect of G-CSF and GM-CSF that was shown by us further supports this discrepancy between proliferation rate and tumor invasion/malignancy. Indeed, GM-CSF and G-CSF up-regulate the expression of MMP-2 and membrane type 1-MMP in human HNSCC cells (24, 46) and urokinase-type plasminogen activator in lung cancer cells (7) and may thus contribute to tumor invasion and angiogenesis.

(b) Accordingly, the malignancy-promoting effect of G-CSF and GM-CSF is substantiated by the enhanced and persistent angiogenesis in surface transplants of factor-expressing tumors. Onset of angiogenesis was early followed by vessel penetration into the tumor, resulting in a highly vascularized tumor tissue. Only very few vessels were associated with α-smooth muscle actin–positive perivascular cells. In contrast, in factor-negative tumors, angiogenesis was delayed by 1 to 2 weeks and transient with reduction in mean vessel density in late transplants and lack of vessel penetration into the tumor mass. The number of matured vessels associated with α-smooth muscle actin–positive cells was tripled compared with factor-expressing tumors. This phenotype was not correlated with VEGF or bFGF expression in the tumors (23). Correspondingly, we previously showed the angiogenesis- and progression-promoting effect of G-CSF and GM-CSF in the HaCaT model of skin SCC (6). Angiogenesis has long been established as critical contribution to tumor growth and progression (47). Recently, we and others could show that blockade of persistent angiogenesis resulted in blood vessels maturation, normalization of the activated tumor stroma, and, ultimately, abrogation of tumor
invasion (48–50). As tumor angiogenesis is regulated by the disbalanced activity or expression of angiogenic stimulators and inhibitors (51), our data place G-CSF and GM-CSF among the stimulators of tumor angiogenesis in vivo.

(c) In addition to angiogenesis, the infiltration of leukocytes is a critical component of the activated tumor-promoting stroma (3, 52) and G-CSF and GM-CSF are known to modulate recruitment, proliferation, and maturation of granulocytes and macrophages (11). We showed previously that expression of both factors induced enhanced and persistent neutrophil granulocyte and macrophage recruitment into the stroma of skin SCC (6). Accordingly, we observed an earlier, enhanced, and persistent recruitment of neutrophils and macrophages in HNSCC tumors expressing G-CSF and GM-CSF, whereas neutrophil recruitment was delayed and transient with reduction in neutrophil number in late tumor stages of factor-negative tumors. Interestingly, neutrophil recruitment somewhat preceded the onset of angiogenesis and followed similar kinetics, either persistent or transient, dependent on the tumor phenotype and the expression or lack of G-CSF and GM-CSF.

The tumor-promoting or tumor-inhibiting role of the inflammatory infiltrate is still a matter of controversial discussion. However, recent reports suggest that inflammatory cells and cytokines are more likely to contribute to tumor growth and progression than to mount an effective host antitumor response (41, 52, 53). There is increasing evidence that macrophages play a critical role in modulating a protumor or antitumor response. Mediated by specific cytokines in their environment, they can differentiate either into a type 1 macrophage mounting an antitumor immune response or into a protumor type 2 macrophage that secretes angiogenic factors and proteases, supporting tumor growth and invasion (54). In >80% of clinical tumors, high macrophage content correlates with poor prognosis (55), and elevated blood levels of hematopoietic cytokines without overt inflammation are associated with worse patient prognosis (22, 56, 57). Tumors recruit granulocytes and macrophages, coopting their abilities for matrix remodeling and angiogenesis by secretion of angiogenic factors and proteases for tumor progression (52, 53, 55, 58). This is evident by mast cell–derived MMP-9 in a mouse model of human papillomavirus–induced skin

Figure 6. Kinetics of macrophage recruitment in surface transplants of HNSCC tumor cells. Immunofluorescent staining for keratin (green), macrophages (red), and nuclei (blue) of cryosections from surface transplants (7, 14, and 28 days) of G-CSF- and GM-CSF-expressing HNO199(+) (A) and factor-negative HNO206(-) (B) cells. G-CSF and GM-CSF expression induced an earlier and enhanced macrophage recruitment. Bar, 100 μm. C, quantification of macrophages in the tumor stroma and in the tumor mass of HNSCC surface transplants. The number of macrophages in the stroma of G-CSF- and GM-CSF-expressing tumors was increased and invasion of macrophages into the tumor mass was drastically enhanced. *, P < 0.05.
carcinogenesis (59) and the neutrophil-mediated MMP-9 secretion in the stroma of malignant SCC (48–50). In agreement with this, the infiltration of neutrophil granulocytes into the tumor tissue was shown to be important for benign tumor cells to acquire a metastatic phenotype (60). Finally, leukocytes can generate reactive oxygen radicals that induce DNA damage, resulting in genomic alterations that further promote malignant conversion (61). Thus, as a critical part of the activated tumor stroma, neutrophils and macrophages promote malignancy by multiple direct or indirect mechanisms. As we have shown, G-CSF and GM-CSF contribute to these effects by inducing their enhanced and persistent recruitment into the stroma of HNSCC. In an immunosuppressed background, both factors seem to be sufficient to induce a protumor inflammatory infiltrate similar to the one observed for highly malignant skin SCC in immunocompetent mice.

Takeda, our results show a decisive role for the coexpression of G-CSF and GM-CSF and their receptors in promoting tumor progression in HNSCC via multiple autocrine and paracrine mechanisms: G-CSF and GM-CSF expression induces an autocrine stimulation of tumor cell proliferation and migration in vitro; they are crucial for tumor invasion in the three-dimensional organotypic culture and induce a paracrine activation of the tumor stroma resulting in enhanced and persistent angiogenesis and recruitment of inflammatory cells in vivo. To further confirm their functional role, we have stably transduced G-CSF and GM-CSF into a factor-negative HNSCC tumor cells. Preliminary results confirm the progression-promoting effects of the factors.5

Questions concerning a role of G-CSF and GM-CSF in tumor progression have become of great clinical interest because both factors are widely used in cancer therapy to ameliorate mucositis and neutropenia and to accelerate wound healing. This use relies on data showing no adverse effect of both factors (26) and even suggesting that the potential proliferation-promoting effect of G-CSF and GM-CSF might increase cancer cell susceptibility to radiation and chemotherapy (31). However, a more recent trial has ascertained that prophylactic G-CSF administration worsens prognosis in HNSCC (33). Thus, despite ameliorating mucositis, neutropenia, and wound healing, G-CSF or GM-CSF are not associated with improved cancer control or survival in this tumor entity but might rather impair disease control. Our data as well as other studies clearly show a functional role of G-CSF and GM-CSF in tumor progression (4, 6, 7), suggesting this as a potential mechanism for the observed adverse effects of adjuvant G-CSF therapy in HNSCC.

On the one hand, these data make it highly desirable to test the actual contribution of exogenously administered G-CSF and GM-CSF to tumor progression. Additionally, the importance of G-CSF and GM-CSF in the autocrine stimulation of tumor cells as well as for tumor invasion and metastasis makes them promising new targets for new therapeutic approaches directed against both tumor cells and the activated tumor supporting stroma.

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