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Cooperative Autocrine and Paracrine Functions of Granulocyte Colony-Stimulating Factor and Granulocyte-Macrophage Colony-Stimulating Factor in the Progression of Skin Carcinoma Cells

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ABSTRACT

Tumor growth and progression are critically controlled by alterations in the microenvironment often caused by an aberrant expression of growth factors and receptors. We demonstrated previously that tumor progression in patients and in the experimental HaCaT tumor model for skin squamous cell carcinomas is associated with a constitutive neoexpression of the hematopoietic growth factors granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), causing an autocrine stimulation of tumor cell proliferation and migration *in vitro*. To analyze the critical contribution of both factors to tumor progression, G-CSF or GM-CSF was stably transfected in factor-negative benign tumor cells. Forced expression of GM-CSF resulted in invasive growth and enhanced tumor cell proliferation in a three-dimensional culture model *in vitro*, yet tumor growth *in vivo* remained only transient. Constitutive expression of G-CSF, however, caused a shift from benign to malignant and strongly angiogenic tumors. Moreover, cells recultured from G-CSF–transfected tumors exhibited enhanced tumor aggressiveness upon reinjection, *i.e.*, earlier onset and faster tumor expansion. Remarkably, this further step in tumor progression was again associated with the constitutive expression of GM-CSF strongly indicating a synergistic action of both factors. Additionally, expression of GM-CSF in the transfected tumors mediated an earlier recruitment of granulocytes and macrophages to the tumor site, and expression of G-CSF induced an enhanced and persistent angiogenesis and increased the number of granulocytes and macrophages in the tumor vicinity. Thus both factors directly stimulate tumor cell growth and, by modulating the tumor stroma, induce a microenvironment that promotes tumor progression.

INTRODUCTION

Tumors arise as the result of a sequence of events leading to tumor growth and subsequent tumor progression. The latter is characterized by an increasing escape of tumor cells from the regulatory influences of their microenvironment. This is often the result of an unregulated expression of growth factors (for review, see refs. 1 and 2), stimulating tumor cell proliferation and/or stromal activation and angiogenesis through autocrine or paracrine loops with the appropriate receptor-bearing cells (3).

Among these aberrantly expressed factors are the hematopoietic growth factors granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), which were originally identified as factors controlling proliferation, maturation, and functional activity of granulocytes and macrophages (4). Both G-CSF and GM-CSF are highly glycosylated proteins of 30- and 22-kDa, respectively (5–7). Human G-CSF exists in two functionally active splice forms, which differ by a deletion of 3 amino acids in the 5′ region of the second intron (8). Its cognate receptor exists in five isoforms with differences in the cytoplasmic domain (9). GM-CSF binds to a dimeric receptor with a ligand-specific α-subunit and a β-subunit that is shared with the receptors for interleukin (IL)-3 and IL-5 (5, 10).

Beside their function as growth and differentiation factors of the hematopoietic system, G-CSF and GM-CSF were also described to be expressed by fibroblasts (5, 11–13), endothelial cells (5, 12), and keratinocytes (12). Both factors are well established as inducers of endothelial cell proliferation and migration *in vitro* and as stimulators of angiogenesis *in vivo* (14, 15). GM-CSF additionally plays an important role as a growth and differentiation factor in normal skin. After induction by keratinocyte-derived IL-1, dermal fibroblasts produce GM-CSF, which in turn, in a double paracrine mechanism, stimulates keratinocyte growth and differentiation (13). Furthermore, upon induction by appropriate stimuli, such as IL-1, tumor necrosis factor (TNF)-α, or lipopolysaccharides, *e.g.,* in a wound situation, keratinocytes are capable of secreting GM-CSF themselves (13).

Recently, G-CSF and GM-CSF have gained increasing attention as factors that are aberrantly expressed in a number of different solid tumors. G-CSF has been described to be newly expressed in squamous cell carcinomas (SCCs) of the esophagus (16) and tongue (17), carcinosarcomas (18), and head and neck carcinomas (19). Constitutive expression of G-CSF and GM-CSF together has been found in SCCs (11, 12, 20), osteosarcoma (21), gliomas (5), meningiomas, and pulmonary adenocarcinoma (22). Frequently, expression of G-CSF and GM-CSF by tumor cells is associated with a coexpression of the respective receptors, and there are first indications that this factor-receptor coexpression may lead to an autocrine stimulation of tumor cell growth, migration, invasion, and metastasis: *e.g.,* G-CSF and GM-CSF stimulate proliferation and migration of SCCs of the skin and gliomas (5, 11, 12); GM-CSF enhances proliferation in renal cell carcinoma (23); expression of G-CSF is associated with more aggressive tumor growth in cervical cancer (24) and enhanced invasion and metastasis in head and neck tumors (9, 25).[1] In addition to this autocrine effect on the cytokine-producing tumor itself, G-CSF and GM-CSF may also act in a paracrine manner on the tumor-surrounding stroma, *e.g.,* by promoting an angiogenic response (14, 15, 26, 27). Furthermore, constitutive expression of G-CSF has been shown to be associated with leukocytosis (16–19) and better neutrophil survival (22). Expression of GM-CSF in SCCs of the head and neck stimulates the recruitment of CD34+ cells, resulting in host immune suppression (27). Interestingly, the recruitment of inflammatory cells to the tumor vicinity has been implicated in the potentiation of neoplastic progression via the production of paracrine factors and could therefore have a strong impact on tumor progression (28).

Thus, one can hypothesize that G-CSF and GM-CSF may contribute to tumor progression not only by acting on the tumor cells themselves but also through activating and/or modulating effects on the tumor stroma and/or the entire organism. Although the mechanis-

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tic basis of these modulating effects remains largely unknown, the clinical relevance of G-CSF– and GM-CSF–mediated effects for patient prognosis becomes increasingly manifest. In studies on oral and nasopharyngeal carcinoma as well as in ovarian carcinomas, expression of G-CSF receptor by the tumor was associated with a worse prognosis and higher relapse rate (29, 30). To better understand the mechanisms by which G-CSF and GM-CSF contribute to tumor growth and progression, we studied their expression and functional effects in an experimental model for human SCCs of the skin, based on the immortal keratinocyte cell line HaCaT and its tumorigenic HaCaT-ras clones (31). In this multistep model for skin carcinomas, progression of tumorigenic HaCaT-ras clones to more aggressive and eventually metastatic phenotypes was reproducibly achieved after their *in vivo* growth as subcutaneous tumors in nude mice and recultivation of tumor cells from these tumors (32). Associated with this *in vivo* progression to an enhanced malignant tumor phenotype, we were able to show a constitutive neoexpression and secretion of G-CSF and GM-CSF in the benign, originally non-expressing tumor cells (12). Because all HaCaT and HaCaT-ras cells produce the receptors for G-CSF and GM-CSF, neoexpression of the respective factors in the enhanced malignant tumor cells resulted in an autocrine stimulation of tumor cell proliferation and migration. Thus, tumor progression was associated with a shift from an originally paracrine stimulation of keratinocyte growth in normal skin to an autocrine stimulatory loop in the keratinocyte tumors (12).

To further clarify the functional role of G-CSF and GM-CSF in tumor progression *in vivo* and their effects on tumor invasion, angiogenesis, and stromal activation, we stably transfected benign originally non-expressing HaCaT-ras cells with vectors containing the coding sequence for G-CSF or GM-CSF. Although cells transfected with G-CSF or GM-CSF showed no growth advantage in monolayer culture *in vitro*, the transfected tumor cells exhibited enhanced proliferation *in vivo*. Constitutive expression of G-CSF in previously benign factor-negative cells resulted in fast-growing invasive tumors after a latency period of about 50 days, indicating a significant tumor progression upon G-CSF transfection. Tumor growth was associated with strong angiogenesis and enhanced recruitment of granulocytes and macrophages. GM-CSF transfection resulted in a transient effect on tumor growth, angiogenesis, and leukocyte recruitment, yet the constitutive expression of GM-CSF did not produce invasive tumors *in vivo*. Tumor progression of a G-CSF transfectant by growth as a subcutaneous tumor *in vivo* and subsequent recultivation of the tumor cells resulted in a further increase in their malignant potential, leading to rapidly growing, highly invasive tumors without any latency. Remarkably, the recultivated tumor cells showed a *de novo* expression of GM-CSF, suggesting a critical and synergistic role for both factors in tumor progression and again confirming that the *in vivo* microenvironment exerts a selective pressure favoring malignant progression. In our model system, we thus provide the first functional evidence for a contribution of G-CSF and GM-CSF to tumor progression in an *in vivo* environment.

MATERIALS AND METHODS

Cell Lines. Cell lines used were the benign HaCaT-ras cell line A-5 (32) and transfectants derived from it (Fig. 1), containing the eukaryotic expression vector pZeoSV with the coding sequences for either G-CSF (two clones, A-5G12b and A-5G16a), GM-CSF (two clones, A-5GM6 and A-5GM14), or vector alone (A-5Z12). A-5G12bRT1D is one of four cell lines obtained by recultivation of tumor tissue derived from two independent subcutaneous injections of A-5G12b cells into nude mice as described elsewhere (11).

A-5 cells were cultivated in 4× modified Eagle's medium (MEM), 10% fetal calf serum (FCS), and neomycin (200 μg/mL, PAA), and the transfectant cell lines were cultivated in 4× MEM, 10% FCS, neomycin (200 μg/mL; PAA, Colbe, Germany), and zeozin (200 μg/mL; Invitrogen, Carlsbad, CA) as described previously (31). Cells were passaged at a split ratio of 1:6 to 1:10, and they were routinely tested for *Mycoplasma* contamination as described previously (33) and always found to be negative.

Plasmids. Shortly, cDNA fragments of G-CSF or GM-CSF were amplified by polymerase chain reaction (PCR) using specific restriction site-containing primers.

The respective fragments for G-CSF or GM-CSF were ligated into the multiple cloning site of the vector pZeoSV (Invitrogen) and verified by restriction digest and sequence analysis. As control vector in the transfection experiments, pZeoSV without insert was used.

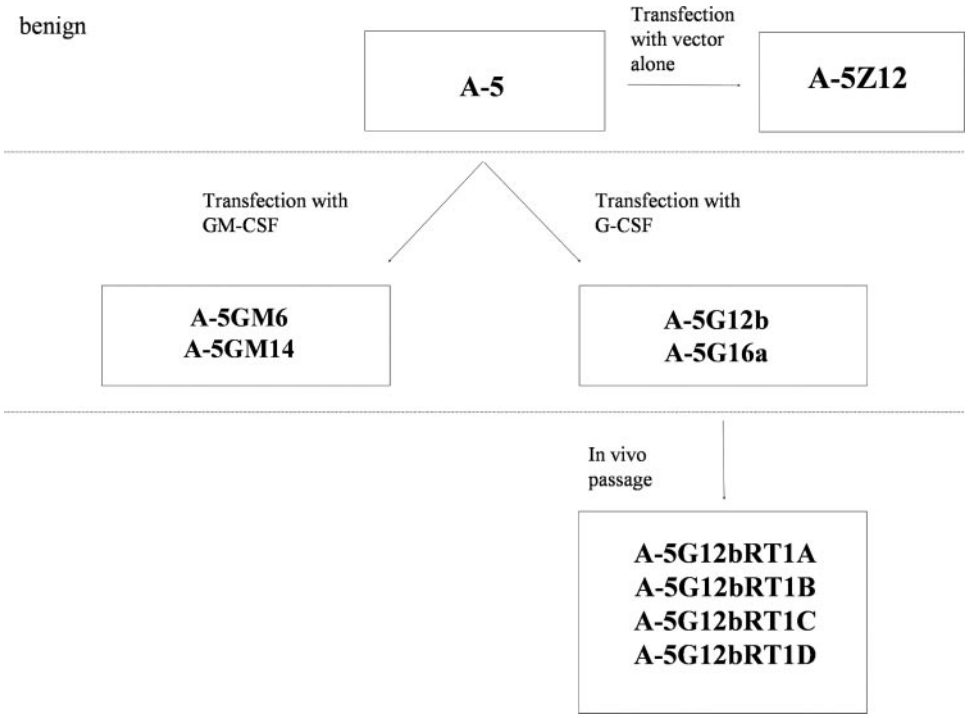

Fig. 1. The origin of the cell lines used was the benign HaCaT-ras cell line A-5 and transfectants derived from it, containing the eukaryotic expression vector pZeoSV with coding sequences for either G-CSF (A-5G12b and A-5G16a), GM-CSF (A-5GM6 and A-5GM14), or vector alone (A-5Z12). A-5G12bRT1A, A-5G12bRT1B, A-5G12bRT1C, and A-5G12bRT1D cells were obtained by recultivation of tumor tissue derived from subcutaneous injection of A-5G12b cells into nude mice.

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Growth Curves. To compare their *in vitro* growth capacities, cells were seeded at a density of 8×10^3 cells per well in 12-well plates and cultivated in 4× MEM containing 10% FCS, neomycin (200 μg/mL), and zeozin (200 μg/mL; for transfectants only). Cell numbers were determined by counting 3 wells per day for 10 days, and experiments were done in duplicate. Data shown are mean values ± SD.

Conditioned Media and Enzyme-Linked Immunosorbent Assay. To generate conditioned media, 2.5×10^3 cells per cm^2 were seeded into 6-cm culture dishes in medium containing 10% FCS. After 24 hours, cells were shifted to medium without FCS. Ninety six hours later, the conditioned medium was harvested, centrifuged for 10 minutes at $10,000 \times g$, and stored in aliquots at −80°C. Enzyme-linked immunosorbent assays for G-CSF and GM-CSF were performed using Quantikine Immunoassay kits from R&D Systems (Minneapolis, MN; human G-CSF, DCS50; human GM-CSF, DGM00) according to the manufacturer's instructions. Samples were tested in duplicate. Data shown are mean values of at least two independent experiments.

Cell Migration Assay. Cells were seeded in 6-well plates at a density of 100,000 cells per well in two replicas. Twenty four hours after the cells had reached confluence, the monolayer was disrupted using a cell scraper of 1 cm in width, and the borders were marked. The culture medium was replaced by serum-free medium containing 50 or 100 ng of either G-CSF or GM-CSF (R&D Systems) or 1 or 2 μg of neutralizing antibodies against G-CSF (clone G61.8.1; Nr.GF14L; Calbiochem, San Diego, CA) or GM-CSF (clone GM4.1.9; Nr. GF13L; Calbiochem), respectively, or an irrelevant antibody (mouse IgG1κ; M-7894; Sigma, St. Louis, MO). Cell migration was documented by microscopic photos taken at time point 0 and after 24 and 48 hours. Migration distance was determined by measuring the photos taken at a magnification of ×200 and calculated back to the actual migration distance. Data shown are the mean of at least three independent experiments with two replica platings each.

Tumorigenicity Assays *In vitro*: Organotypic Cocultures. Dermal equivalents for organotypic cocultures were prepared with native type I bovine collagen. The lyophilized collagen was redissolved with 0.1% acetic acid to a final concentration of 4 mg/mL. Eight volumes of ice-cold collagen solution were mixed with 1 volume of 10× Hanks' buffered saline followed by neutralization with 2 mol/L NaOH. One volume of FCS was added and mixed thoroughly, resulting in a final concentration of 3 mg/mL. Of this mixture, 2.5 mL each were poured into polycarbonate membrane filter inserts (Falcon No. 3501; Becton Dickinson, Heidelberg, Germany), placed in special deep 6-well trays (Becton Dickinson), and allowed to gellify at 37°C. Glass rings (24-mm outer diameter; 20-mm inner diameter) were put onto the gels to compress them and provide a flat, central area for tumor cell seeding. The gels were equilibrated with 4× MEM, 10% FCS, and 50 μg of L-ascorbic acid (Sigma) per mL. Tumor cells (8.5×10^5) were seeded on top of the collagen matrix. After submersed incubation overnight, the cultures were raised to the air-medium interface by lowering the medium level. For three weeks, two cultures per week were taken out and processed for cryostat sectioning. Data shown are representative of three independent experiments.

Tumorigenicity Assays *In vivo*. Tumor formation was assayed by subcutaneous injection of 5×10^6 cells in a final volume of 100 μL into the interscapular region of 4 -to 6-week–old athymic nude mice. The growth of the resulting tumors was monitored by measuring tumor size in two axes and calculating the tumor volume following published procedures (34). If a tumor had reached a size of up to 1 cm^2, it was taken out and further processed for histology and cryostat sectioning.

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 I rat collagen gel (4 mg/mL) mounted between two concentric Teflon rings (Renner, Dannstadt, Germany). Before transplantation, the chamber was covered with a silicon hat and then transplanted onto the dorsal muscle fascia of 6-week–old nude mice as described previously (31). For 6 weeks, three transplants per week were dissected and processed for cryostat sectioning.

Recultivation of Tumor Cells. To recultivate tumor cells, vital tumor segments were removed, minced into pieces of 1 to 2 mm in size, placed on a culture dish precoated with FCS, and cultivated with 4× MEM and 10% FCS. After formation of big islands of keratinocytes, neomycin (200 μg/mL) and zeozin (200 μg/mL) were added to the culture medium to free recultivated transfectants from contaminating (mouse) cells and maintain selection for the transfected H-ras and G-CSF cDNA. Four tumor cell lines were recultivated from two independent subcutaneous injections yielding similar results. Data are shown for one representative cell line, A-5G12bRT1D.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction. RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA), following the manufacturer's instructions. Reverse transcription-polymerase chain reaction (RT-PCR) was carried out with the Gene Amp RNA PCR Core Kit (Perkin-Elmer, Wellesley, MA). Reverse transcription was performed in a volume of 100 μL, using 5 μg of RNA; 5 mmol/L $MgCl_2$; 1× PCR buffer; 1 mmol/L dATP, dGTP, dCTP, and dTTP; 1 unit/μl RNase inhibitor; 2.5 units/μl murine leukemia virus reverse transcriptase; 1.25 μmol/L random hexamer primer; and 1.25 μmol/L oligo(dT) primer and double-distilled H_2O. PCR reactions contained 9 μL of the reverse transcription reaction and 2.5 units of AmpliTaq DNA polymerase in a volume of 100 μL. $MgCl_2$ concentration and annealing temperature were optimized for each primer set. PCR conditions for cloning were as follows: (*a*) G-CSF: 1.5 mmol/L $MgCl_2$, 35 cycles of 95°C for 1 minute, 63°C for 1 minute, and 72°C for 2 minutes; and (*b*) GM-CSF: 1.5 mmol/L $MgCl_2$, 30 cycles of 94°C for 1 minute, 59°C for 1 minute and 30 seconds, and 72°C for 1 minute and 30 seconds. RT-PCR conditions were as follows: (*a*) G-CSF: 2.75 mmol/L $MgCl_2$, 35 cycles of 94°C for 1 minute, 70°C for 1 minute and 30 seconds, and 72°C for 1 minute and 30 seconds; (*b*) G-CSF receptor: 2 mmol/L $MgCl_2$, 35 cycles of 94°C for 1 minute, 60°C for 1 minute and 30 seconds, and 72°C for 1 minute and 30 seconds; (*c*) GM-CSF: 2 mmol/L $MgCl_2$, 25 cycles of 94°C for 1 minute and 30 seconds, 60°C for 2 minutes, and 72°C for 3 minutes; and (*d*) GM-CSF receptor β: 2 mmol/L $MgCl_2$, 35 cycles of 94°C for 1 minute, 60.5°C for 1 minute and 30 seconds, and 72°C for 1 minute and 30 seconds.

Oligonucleotide Primers. Sense and antisense primers were synthesized according to the sequences extracted from GenBank. The primers used for cloning were as follows: (*a*) G-CSF, 5′-ggccatgcat (Nsi site) and 3′-ggcctctaga (Xba site); and (*b*) GM-CSF, 5′-ggccggatcc (*Bam*HI site) and 3′-ggccggtacc (*Kpn*I site). The primers used for RT-PCR were as follows: (*a*) G-CSF: (bp 93–118) 5′-cacagtgcactctggacagtgcagg, and (bp 479–508) 3′-tagaccgtcgtctac-cttcttgacccttac; (*b*) G-CSF receptor: (bp 1141–1160) 5′-cctggagctgagaactaccg, and (1431–1450) 3′-gccaccagaagagtctttcg; (*c*) GM-CSF: (bp 42–62) 5′ tggc-ctgcagcatctctgca, and (bp 344–364) 3′-acacgttgggtctgatagtg; and (*d*) GM-CSF receptor β: (bp 981-1000) 5′-aatacatcgtctctgttcag, and (bp 1297–1317) 3′-tcactccactcgctccagat.

All oligonucleotide primers spanned intron–exon splice sites, ensuring that PCR products generated from any DNA present in the RNA preparations could be clearly distinguished from those generated in RT-PCRs. The identity of the PCR amplification products was confirmed by size and restriction digest and/or sequencing.

Indirect Immunofluorescence. Six-micrometer cryosections of frozen tumors were mounted on slides, air dried, and stored at −80°C. Cryosections were fixed for 10 minutes in acetone at −20°C, air dried, and washed in PBS+ (Serva). Slides were blocked with 12% bovine serum albumin for 15 minutes, and the primary antibody was directly applied. Alternatively for antibodies derived from mice, sections were incubated with goat antimouse IgG(h+l) Fab fragment (Dianova) for 1 hour and washed in PBS+ before applying the primary antibody. Slides were incubated for 90 minutes with the primary antibody at room temperature and then washed and incubated with the fluorescent secondary antibody for 1 hour, washed, and mounted. Primary antibodies used were as follows: (*a*) pan-cytokeratin, guinea pig, polyclonal antibody (Progen, Heidelberg, Germany; clone GP14; 1:100 dilution); (*b*) murine CD31, rat monoclonal antibody (PharMingen, San Diego, CA; no. 01951D; 1:100 dilution); (*c*) pan-macrophages, rat monoclonal antibody (Dianova, Hamburg, Germany; no. D-2006; 1:50 dilution); (*d*) murine neutrophils, rat (Serotec, Duesseldorf, Germany; no. MCA771G; 1:100 dilution); (*e*) human collagen IV rabbit, polyclonal antibody (Heyl, Berlin, Germany; no. 302001000; 1:100 dilution); and (*f*) Ki67 (mib), mouse monoclonal antibody (Dianova; no. Dia 505; 1:20 dilution). Secondary antibodies used were as follows: (*a*) anti-guinea pig, donkey, DTAF (Dianova; no. 706-015-148; 1:100 dilution); (*b*) antimouse goat Texas Red (Dianova; no.115-076-062; 1:200 dilution); (*c*) antirat donkey Texas Red (Dianova; no. 712-076-153; 1:200 dilution); and (*d*) antirabbit goat Texas Red (Dianova; no. 111-076-045; 1:200 dilution).

Quantification. For quantifications, photos of three immunofluorescence stainings of three animals for each time point were analyzed using the Analysis Software (Schärfe System, Reutlingen, Germany).

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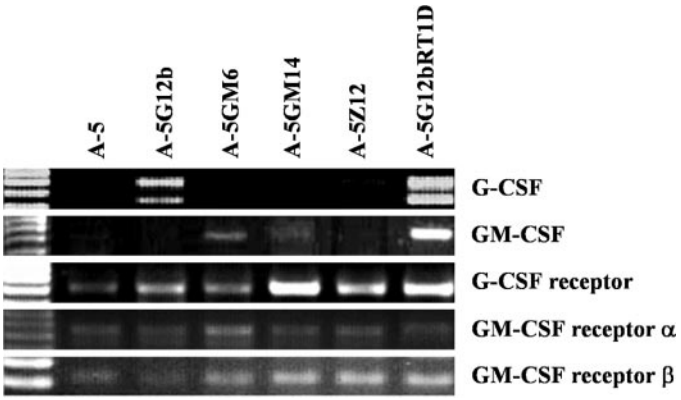

Fig. 2. Expression of G-CSF (417 bp), GM-CSF (322 bp), G-CSF receptor (310 bp), and GM-CSF receptor α (548 bp) and receptor β (337 bp), as determined by RT-PCR (DNA marker, perfect DNA 100-bp ladder; Novagen).

Statistical Analysis. For statistical analysis, a two-tailed Mann-Whitney test was performed using GraphPad Prism 4.0a for Macintosh (San Diego, CA). $P < 0.05$ was considered significant.

RESULTS

Transfection with G-CSF or GM-CSF and Expression of Factors and Their Receptors. Benign HaCaT-ras A-5 cells that do not express either G-CSF or GM-CSF but express the respective growth factor receptors (12) were transfected with a G-CSF or GM-CSF cDNA expression plasmid or with vector alone, using zeozin as a selection marker. Clonal transfected populations were isolated, and positive cell clones were identified by PCR and RT-PCR, demonstrating the presence of the intact plasmid in the cellular DNA and mRNA expression of the exogenous G-CSF and GM-CSF. After transfection, the two G-CSF–expressing cell lines A-5G12b and A-5G16a and the two GM-CSF–expressing cell lines A-5GM6 and A-5GM14 (Fig. 1) were selected for additional experiments. Both G-CSF—expressing cell lines and both GM-CSF–expressing cell lines yielded similar results. Parental A-5 cells as well as the control transfectant A-5Z12 do not express both factors (Fig. 2). On the other hand, the G-CSF transfectants (A-5G12b and A-5G16a) and the GM-CSF transfectants (A-5GM6 and A-5GM14) express the respective factors at the mRNA (Fig. 2) and protein level (Table 1). Interestingly, the amplified G-CSF mRNA fragments in the clone A-5G12b appear as a doublet of bands of 417 and 406 bp, the latter of which results from a previously described G-CSF splice variant (8). Sequence analysis of the third fragment of 258 bp revealed a form of G-CSF with a deletion of 159 nucleotides (Fig. 2). The functionality of this fragment was not determined. Protein expression levels between the clones differed slightly but not significantly and were within the range seen previously for the enhanced malignant A-5RT1 and A-5RT3 cells (ref. 12; Table 1).

To confirm the maintenance of the G-CSF and GM-CSF receptor expression in the transfected clones and thus the potential to establish an autocrine stimulatory loop on coexpression of factor and receptor, we analyzed the expression of G-CSF, GM-CSF, and their receptors via RT-PCR. In agreement with the expected expression profile, all cell lines tested expressed the G-CSF and GM-CSF receptor, as evidenced by amplification of the 310- and 337-bp fragment, respectively (Fig. 2). Data shown here will concentrate on A-5G12b, A-5GM6, and A-5GM14.

Influence of G-CSF and GM-CSF on Tumor Cell Growth *In vitro*. Because G-CSF and GM-CSF are known to stimulate the proliferation of keratinocytes *in vitro*, their potential autocrine influence on tumor cell growth *in vitro* was analyzed in monolayer cultures containing 10% FCS or 0% FCS. Comparison of G-CSF and GM-CSF transfectants, control transfectant, and parental cell line revealed no significant difference in *in vitro* growth behavior in monolayer culture in both serum concentrations, indicating that the constitutive expression of both factors does not result in a growth advantage under these conditions (Fig. 3*A*, data shown for 10% FCS).

Nevertheless, G-CSF– or GM-CSF–expressing cells exhibited a profound growth advantage when grown in three-dimensional culture on a collagen gel at the air–liquid interface *in vitro*. In this assay, expression of G-CSF or GM-CSF was associated with enhanced proliferative activity of the tumor cells, as demonstrated by staining for the proliferation-associated protein Ki-67 (Fig. 3*B*−*D*) and by a significant infiltration into the collagen gel (Fig. 3*C*−*F*).

Modulation of Tumor Cell Migration by G-CSF and GM-CSF. Because G-CSF and GM-CSF are also known to modulate tumor cell migration (5, 12), we investigated their influence on the migration of benign, nontransfected A-5 cells and control-transfected A-5Z12 cells as well as on the factor-expressing transfected clones A-5G12b and A-5GM6. There was no significant difference in the unstimulated migration capacity of parental A-5 cells and growth factor-transfected clones (Fig. 4). In agreement with our previous findings for enhanced malignant tumor cells (11, 12), G-CSF and GM-CSF strongly modulate tumor cell migration *in vitro*, and coexpression of factor and receptor results in an autocrine regulatory loop. As a consequence, migration of A-5 cells and control transfectants (which do not express either factor) was significantly ($P < 0.05$) stimulated (189% or 187%, respectively) by the addition of 50 or 100 ng/mL G-CSF or GM-CSF (Fig. 4*A*). In accordance with this, and similarly to the autocrine effect on tumor cell growth, migration of the A-5G12b and A-5GM14 cells was significantly inhibited by 27% ($P < 0.05$) or 87% ($P < 0.01$) in the presence of 2 μg/mL neutralizing antibodies against G-CSF and GM-CSF, respectively (Fig. 4*B* and *C*). Interestingly, the anti–GM-CSF antibody exhibited a more pronounced effect concomitant with the higher absolute amount of factor expressed in the GM-CSF transfectant when compared with the G-CSF transfectant. Control experiments with an irrelevant antibody or with medium alone showed no effect (data not shown).

Tumor Growth *In vivo*. To determine whether this effect of G-CSF and GM-CSF on tumor cell proliferation and migration *in vitro* was mirrored by a similar influence on tumor growth *in vivo*, 5×10^6 cells of the growth factor-transfected cell lines and the control cell lines A-5 and A-5Z12 were injected subcutaneously into nude mice (Fig. 5).

Parental benign A-5 cells and A-5Z12 control transfectants produced small cystic tumor nodules that appeared after a very long latency period of 70 to 120 days in 2 of 8 and 2 of 10 injections, respectively. These nodules consist of a rim of squamous epithelia

Table 1 *Protein expression of G-CSF and GM-CSF*

	A-5	A-5RT1	A-5RT3	A-5G12b	A-5G16a	A-5GM6	A-5GM14	A-5Z12
Human G-CSF (pg/mL)	∅	94.5 ± 3.5	25 ± 7.07	101 ± 6.5	162 ± 18	∅	∅	∅
Human GM-CSF (pg/mL)	∅	927 ± 155	300 ± 60	∅	∅	288 ± 23	113 ± 23	∅

NOTE. Messenger RNA expression data of both factors was confirmed at the protein level using ELISAs for G-CSF and GM-CSF with conditioned media of all cell lines. ∅, protein below detection level.

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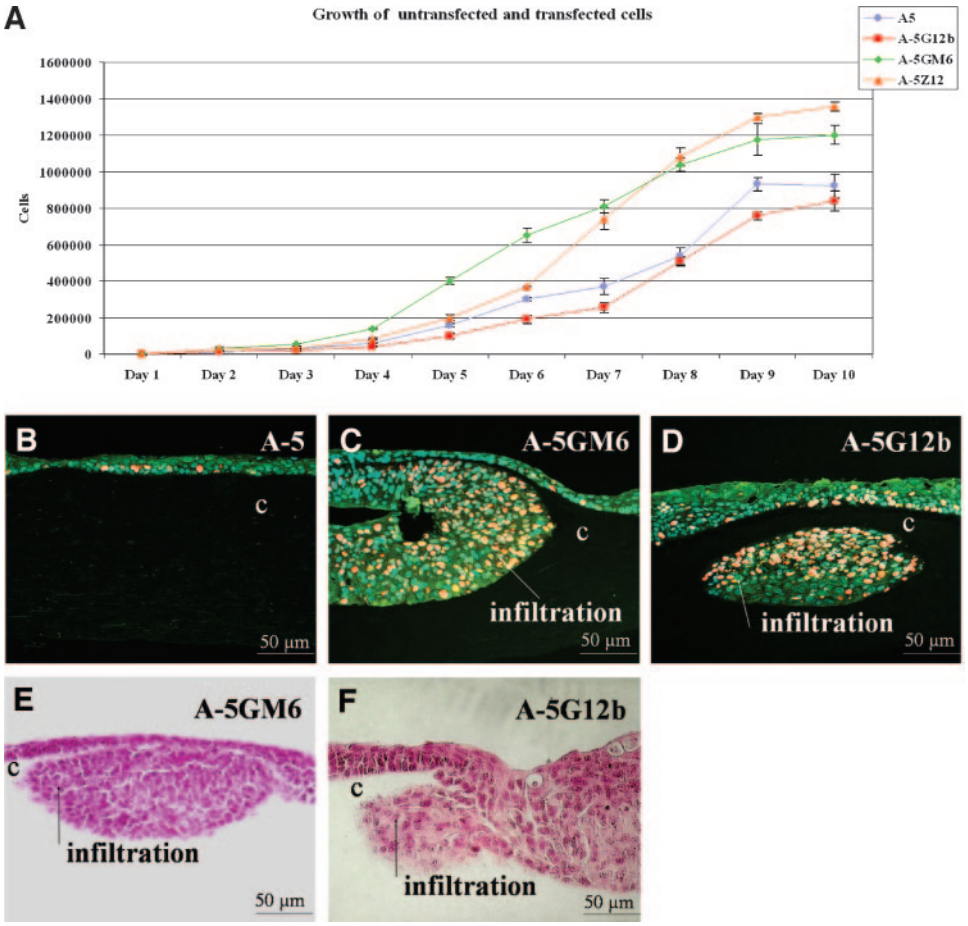

Fig. 3. Influence of G-CSF and GM-CSF on tumor cell proliferation *in vitro*. *A*, cells were seeded at a density of 8×10^3 cells per well in 12-well plates and cultivated in 4× MEM containing 10% FCS. Cell numbers were determined by counting 3 wells per day for 10 days. *B*–*D*. Cell proliferation was determined in 3-week–old organotypic cocultures *in vitro*. Paraffin slides were stained with antibodies against keratin (*green*) and the proliferation-associated protein Ki-67 (*red*); nuclei are stained with Hoechst dye (*blue*). *B*, A-5; *C*, A-5GM6; *D*, A-5G12b. *E* and *F*, tumor cell infiltration (as marked by *arrow*) into the collagen gel was analyzed in 2-week–old organotypic cocultures by hematoxylin and eosin staining of paraffin sections. *E*, A-5GM6; *F*, A-5G12b. *c*, collagen gel.

surrounding a keratinized central part. In 4 of 6 and 8 of 10 injections, respectively, no tumor formation was observed (Fig. 5*A* and *B*; Fig. 6*A*).

Injection of GM-CSF–transfected cell lines (data shown for A-5GM6) resulted in transient tumor growth, initially producing enlarging tumor nodules that lasted for about 6 weeks and then receded.

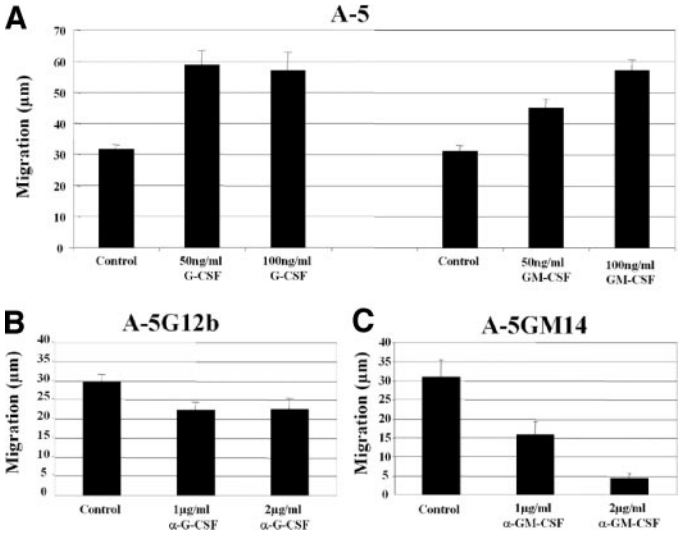

Fig. 4. Influence of G-CSF and GM-CSF on tumor cell migration *in vitro*. Migration was determined in a monolayer scrape assay (*A*) of A-5 cells after stimulation with G-CSF or GM-CSF, (*B*) after incubation of A-5G12b cells with neutralizing antibodies against G-CSF, and (*C*) after incubation of A-5GM14 cells with neutralizing antibodies against GM-CSF for 24 hours. Values shown were significant ($P < 0.05$).

In one of eight injections, a tumor nodule started forming again after 6 months; however, the mouse died of age before any further tumor growth could be observed (Fig. 5*C*).

In contrast, injection of the G-CSF–transfected cell line (A-5G12b) produced fast-growing, histologically invasive tumors in 5 of 10 cases after a latency period of 50 to 70 days (Figs. 5*D* and 6*B*). Compared with the benign, parental, and control tumors, the G-CSF transfectants exhibited enhanced cell proliferation similar to the one seen in the organotypic cultures in Fig. 3 (Fig. 6*E* and *G*).

***In vivo* Tumor Progression of Low-Grade Malignant A-5G12b Cells.** Supporting the important influence of the tumor microenvironment on tumor progression, we previously demonstrated that tumor progression from benign to highly malignant HaCaT-ras SCCs was reproducibly achieved by growth as a subcutaneous tumor in nude mice and subsequent recultivation of tumor cells (11). The novel and constitutive expression of G-CSF and GM-CSF was always associated with this *in vivo* progression (refs. 11 and 12; see Table 1). To determine whether a further tumor progression of the G-CSF–transfected clone could be similarly achieved, tumors induced by A-5G12b cells were recultivated. Four cell lines from two independent subcutaneous injections were established, showing similar results in subsequent experiments. Data are shown for one representative, the cell line A-5G12bRT1D, which exhibited an *in vitro* growth behavior similar to that of the parental cell line A-5G12b (data not shown). *In vivo*, however, the recultivated tumor cells showed a clear progression to an enhanced malignant tumor phenotype, with fast-growing, highly invasive tumors in all animals after a latency period of only 1 to 2 weeks (Figs. 5*E* and 6*C*).

Remarkably, and in agreement with our previous observations,

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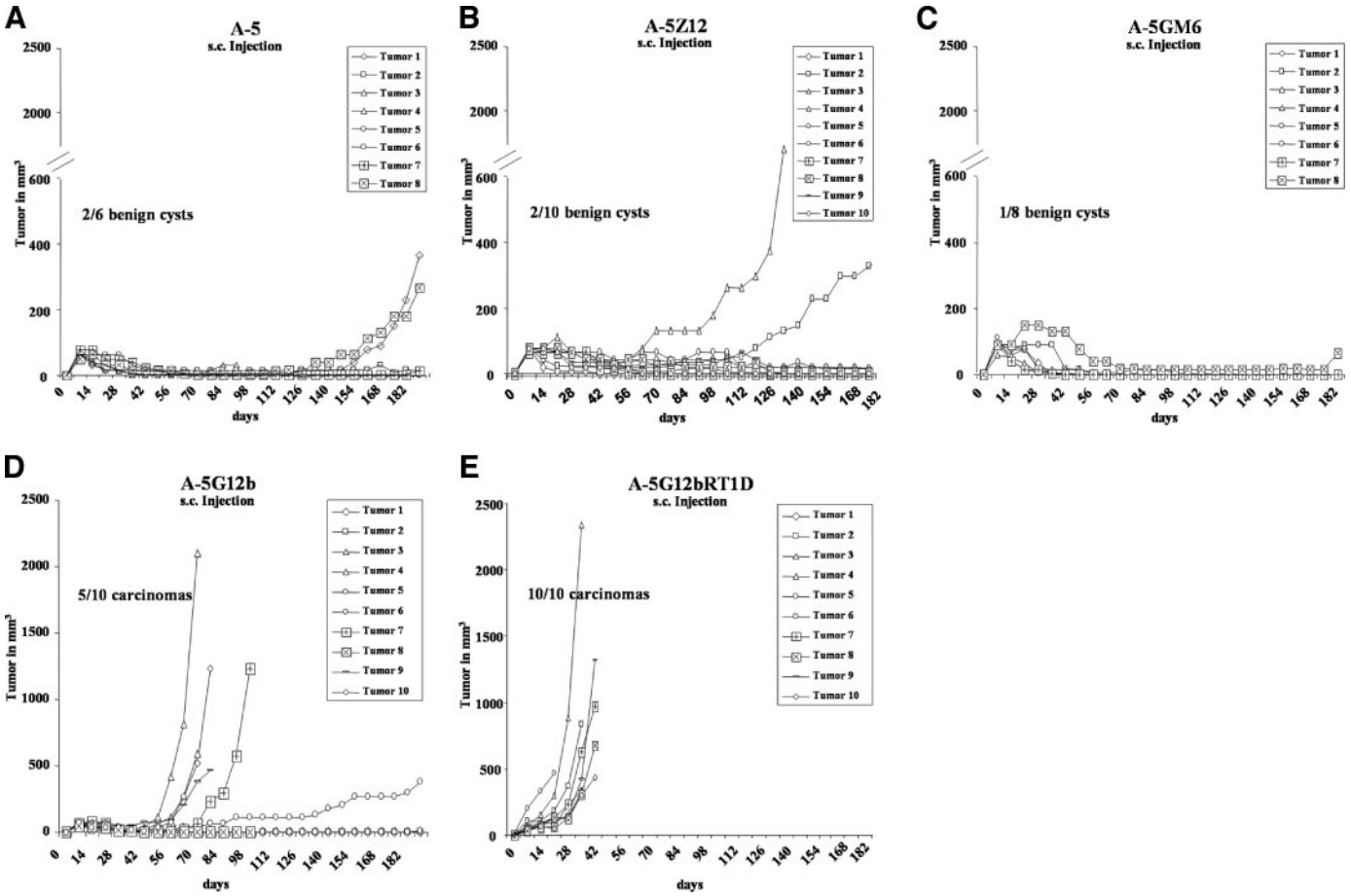

Fig. 5. Tumor growth *in vivo*. Cells (5×10^6) were injected subcutaneously into the nude mouse, and tumor size was monitored at weekly intervals. *A*, A-5 cells (untransfected); *B*, A-5Z12 cells (control transfectant); *C*, A-5GM6 cells (GM-CSF transfected); *D*, A-5G12b cells (G-CSF transfected); *E*, A-5G12bRT1D cells (recultivated G-CSF transfectant).

progression to the enhanced malignant tumor phenotype subsequent to an *in vivo* passage was again associated with the novel and constitutive expression of GM-CSF mRNA in these cells in addition to the transfected G-CSF (Fig. 2). This neoexpression of GM-CSF in the recultivated tumor cells was confirmed at the protein level by enzyme-linked immunosorbent assay, showing a secretion of 106 ± 49 pg/mL, whereas the G-CSF expression remained more or less constant**.**

Tumor Invasion and Angiogenesis. Concomitantly with the enhanced tumor growth *in vivo*, the transfected cell lines and their *in vivo* progressed derivatives showed an enhanced tumor invasion and stromal activation. Subcutaneous tumors of the benign A-5 and control-transfected cells (data not shown) presented as cysts with a multilayered rim of vital tumor cells around a keratinized central part. Around these encapsulated cysts, numerous relatively large blood vessels were observed (Fig. 6*A*). GM-CSF–transfected tumors could not be analyzed histologically because the initially growing tumor nodules regressed completely after 4 to 6 weeks.

In contrast, the G-CSF–transfected cell line A-5G12b formed invasive SCCs that penetrated into the surrounding mouse tissue (Fig. 6*B*), whereas, in turn, blood vessels in stromal strands traversed the tumor parenchyma. This effect was even more pronounced for A-5G12bRT1D cells (Fig. 6*C*), which grew as highly invasive carcinomas with strong proliferative activity in the periphery and large areas of central necrosis (Fig. 6*F* and *G*).

Kinetics of Invasion and Angiogenesis. To study the kinetics of tumor invasion and stromal activation of the parental and transfected cells in more detail and to determine the influence of G-CSF and GM-CSF on these processes, cells were grafted in the surface transplantation assay (35). In this matrix-inserted surface transplantation model, tumor cells are grown on a collagen matrix and transplanted *in toto* onto the back muscle fascia of nude mice. The model allows the observation of early steps in the process of stromal activation, angiogenesis, and invasion because the collagen matrix, while allowing interaction of tumor and host cells via diffusible factors, prevents their immediate contact and intermingling.

Transplants of A-5 cells and control transfectants formed initially thin stratifying layers that expanded until the 4th week. Angiogenesis in these transplants started in week 2 to 3, with blood vessels penetrating into the collagen gel and reaching the tumor-stroma border in week 4. At later time points (*e.g.,* week 6), the lumen of the established vessels increased, indicating vessel maturation concomitantly with a down-regulation of ongoing angiogenesis. No infiltration of vessels into the tumor mass or *vice versa* (invasion of tumor cells into the host stroma) was observed (Fig. 7*A* and *C*).

GM-CSF transfectants (A-5GM6) produced a thin, irregularly structured epithelium without apparent differentiation. Kinetics of angiogenesis were transient and similar to those of the parental and control-transfected cells, with angiogenesis starting in week 2 to 3 (Fig. 7*A* and *C*), reaching a maximum in week 4 when vessels made contact with the tumor tissue followed by a similar vessel maturation as seen in control transfectants (data not shown). As seen in the factor-negative cells, no invasion of blood vessels into the tumor mass or of tumor cells into the surrounding stroma was observed (Fig. 7*C*).

In contrast, in transplants of G-CSF transfectants, angiogenesis started as early as week 1 to 2, with vessels reaching the tumor tissue in week 3, and angiogenesis remaining persistent throughout the

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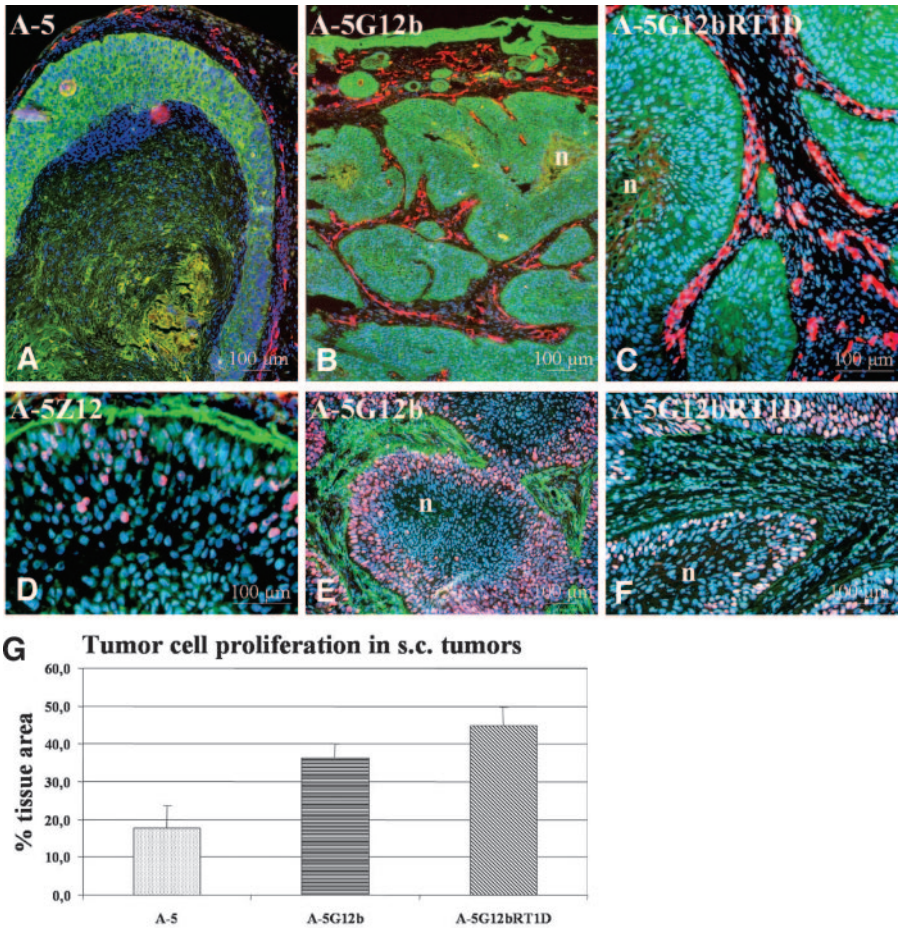

Fig. 6. Cryosections of tumors were fixed with acetone and stained with antibodies against keratin (*green*), CD31 (*red*), and nuclei with Hoechst dye (*blue*) to determine tumor invasion and angiogenesis. *A*, A-5; *B*, A-5G12b; *C*, A-5G12bRT1D and against collagen IV (*green*), the proliferation-associated antigen Ki67 (*red*), nuclei (*blue*) to determine tumor cell proliferation *in vivo*. *D*, A-5Z12; *E*, A-5G12b; *F*, A-5G12bRT1D; G, quantification of proliferating cells shown as a percentage of total cell number; values shown were significant ($P < 0.05$). *n,* necrosis.

observation period (Fig. 7*C*). On contact between tumor tissue and blood vessels, tumor invasion and penetration of blood vessels into the tumor mass started in the 3rd week (*arrows* in Fig. 7*B* and *C*), resulting in vascularized invasive tumor tissue in week 6 (Fig. 7*B*).

In line with their previously observed enhanced malignant phenotype, *in vivo* progressed A-5G12bRT1D cells formed a thicker multilayered epithelium than the G-CSF transfectant after only 1 week (data not shown). Contact between blood vessels and tumor tissue concomitantly with the onset of tumor invasion was already visible in week 2, resulting in a highly vascularized and invasive tumor tissue with persisting angiogenesis at week 4 (Fig. 7*B* and *C*). Thus, the expression of G-CSF is a prerequisite for the induction of a persisting angiogenesis and tumor invasion, whereas the coexpression of GM-CSF stimulated tumor growth, an earlier onset of angiogenesis and invasion, and thus progression to enhanced malignancy.

Recruitment of Granulocytes. G-CSF and GM-CSF are known to stimulate recruitment and differentiation of inflammatory cells such as granulocytes and macrophages, cells that are important players in granulation tissue formation and may contribute to tumor invasion and angiogenesis through the secretion of angiogenic factors and degradation of the extracellular matrix by secreting matrix metalloproteinases (28). In tumor transplants, constitutive expression of G-CSF and GM-CSF indeed resulted in an enhanced recruitment of granulocytes to the tumor stroma. Whereas granulocytes are relatively scarce in the tumor vicinity of subcutaneous tumors of the parental benign A-5 cells and the control transfectant A-5Z12, tumors of the G-CSF transfectant A-5G12b and the *in vivo* progressed A-5G12bRT1D show a tendency to a stronger accumulation of granulocytes (data not shown). Whereas these observations were made at later time points of tumor growth, the kinetics of granulocyte recruitment were analyzed in detail in surface transplants. Benign factor negative cells showed a transient recruitment of granulocytes into the tumor vicinity starting at week 2, increasing and reaching a plateau at week 4 to 5, and decreasing again in number by week 6 (Fig. 8*A* and *C*). In GM-CSF transfectants (shown for A-5GM14) granulocyte recruitment was accelerated starting as early as week 1 yet remained transient as seen in control transplants (Fig. 8*A* and *C*).

However, there was a striking difference in granulocyte recruitment in transplants of the G-CSF transfectant, which were faster and persistent compared to controls, *i.e.*, they reached the tumor border after 2 weeks and further increased and persisted throughout the observation period (Fig. 8*B* and *C*). In the enhanced malignant A-5G12bRT1D tumors, granulocyte recruitment was further accelerated and enhanced, reaching the tumor tissue already in week 1, and granulocyte number subsequently increased to the highest level and remained persistent (Fig. 8*B* and *C*).

Recruitment of Macrophages. Although the number of recruited macrophages in subcutaneous tumors of controls and transfectants showed no obvious difference, detailed analysis in the surface transplants revealed that constitutive expression of GM-CSF resulted in a tendency to an earlier recruitment of macrophages to the tumor site (shown for A-5GM14). This started already at week 1, one week earlier than the parental control, and reached a plateau in week 2 (Fig. 9*A*, *B*, and *E*). G-CSF transfectants (A-5G12b) also showed a slightly earlier macrophage infiltration when compared with controls, reaching a higher cell density well above that of controls and GM-CSF transfectants in weeks 3 and 4. Additionally, in contrast to the controls as well as the

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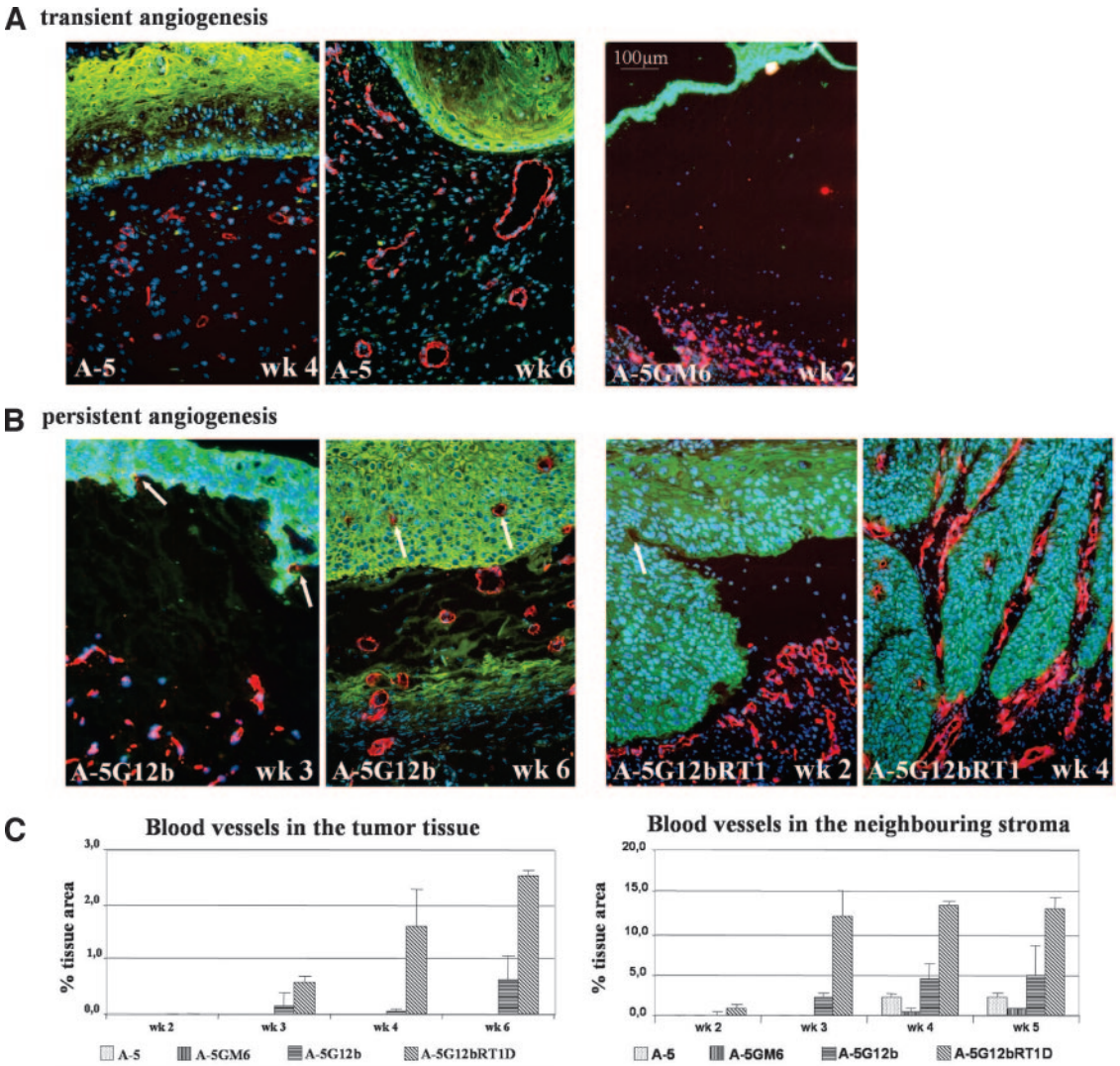

Fig. 7. Kinetics of angiogenesis. Cryosections of surface transplants were fixed with acetone and stained with antibodies against keratin (*green*), CD31 (*red*), and nuclei by Hoechst dye (*blue*) to determine angiogenesis. *A*, A-5, weeks 4 and 6; A-5GM6, week 2. *B*, A-5G12b, weeks 3 and 6; A-5G12bRT1D, weeks 2 and 4 (*arrows* indicate blood vessels in the tumor mass). *C*, quantification of blood vessels in the tumor tissue and in the tumor stroma as a percentage of the tissue area.

GM-CSF transfectants, macrophages infiltrated the tumor mass of G-CSF transfectants (Fig. 9*C*, *arrows*, week 4). Consistent with GM-CSF and G-CSF coexpression in transplants of the *in vivo* progressed A-5G12bRT1D cells, macrophages were visible as early as week 1 and reached a plateau in week 3, indicating similar but accelerated kinetics, as in the G-CSF transfectants (Fig. 9*E*). Again, macrophages strongly infiltrated the tumor tissue (Fig. 9*D*, *arrows*, week 3). Our data therefore indicate an accelerated recruitment of macrophages in response to GM-CSF, whereas the neoexpression of G-CSF seems to contribute to a generally enhanced density of macrophages in the tumor stroma and the infiltration of macrophages into the tumor mass.

DISCUSSION

During tumor progression, alterations that occur in the growth of tumors and the interactions of tumor cells with their stromal environment are frequently associated with the aberrant expression of growth factors and/or growth factor receptors. In the HaCaT model for human SCCs of the skin, we were able to demonstrate a constitutive neoexpression of G-CSF and GM-CSF that is strictly associated with tumor progression to an enhanced malignant tumor phenotype (11) and results in an autocrine stimulation of tumor cell proliferation and migration *in vitro* (12). To analyze the functional contribution of both factors to tumor progression, we transfected benign cells that express the receptors for G-CSF and GM-CSF but not the factors themselves with either G-CSF or GM-CSF, respectively, and analyzed their growth behavior *in vitro* and *in vivo*.

Effects of G-CSF and GM-CSF on Tumor Cells *In vitro*. As demonstrated previously for the enhanced malignant HaCaT-ras tumor cells (12), forced expression of G-CSF or GM-CSF in the receptor-expressing cells (A-5G12b and A-5G16a, A-5GM6, and A-5GM14) establishes an autocrine regulatory loop influencing tumor cell proliferation and migration *in vitro*. In organotypic cocultures, transfection with G-CSF or GM-CSF results in a strongly enhanced tumor cell proliferation as compared with controls. Additionally, in monolayer migration assays, both factors stimulate migration, whereas the presence of neutralizing antibodies inhibits migration. The autocrine growth-regulatory mechanism established by both factors may also function *in vivo*, where it could contribute to tumor progression through an enhancement of cell proliferation and migration and thus of tumor growth, invasion, and metastasis (4, 10, 14, 36).

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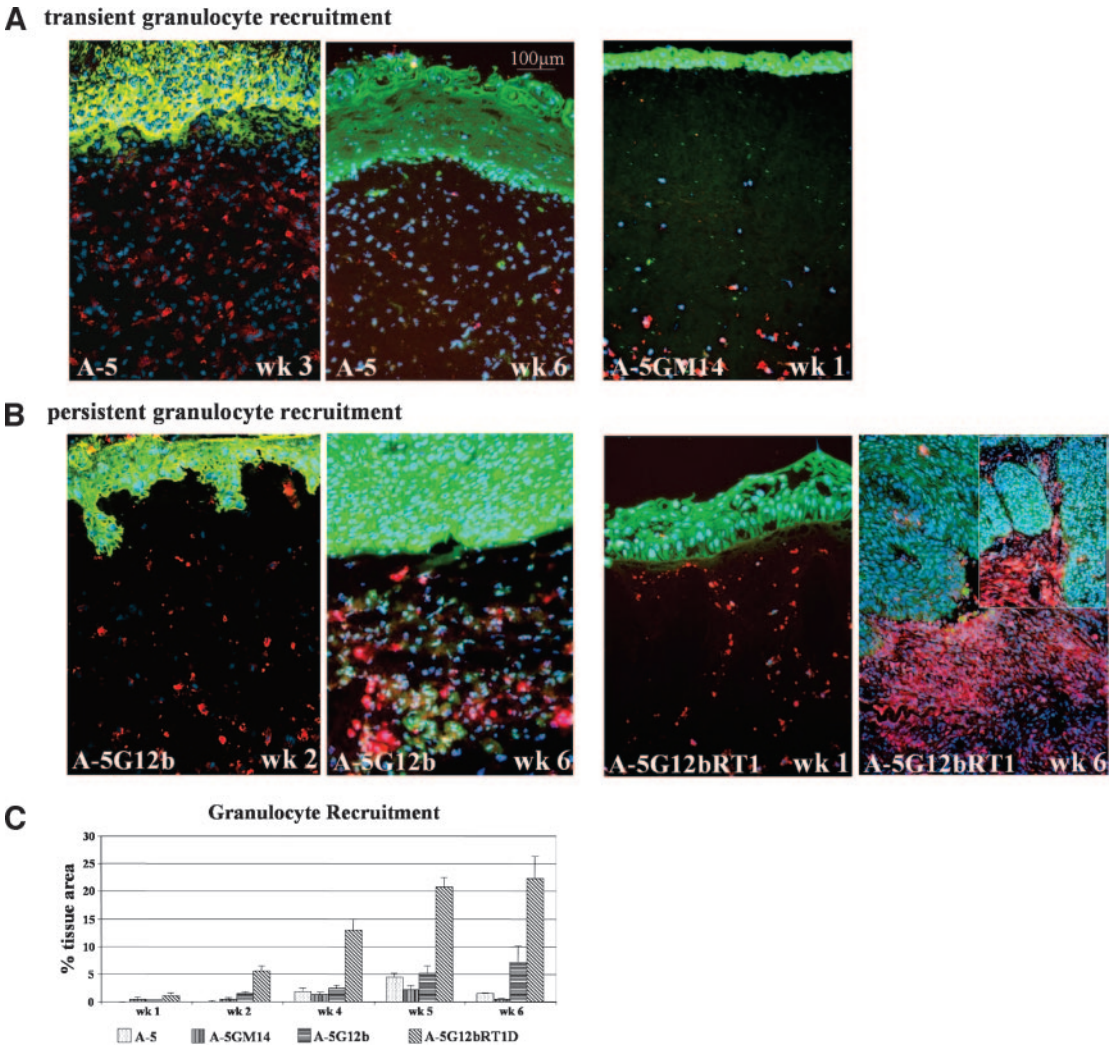

Fig. 8. Kinetics of granulocyte recruitment. Cryosections of surface transplants were fixed with acetone and stained with antibodies against keratin (*green*), granulocytes (*red*), and nuclei by Hoechst dye (*blue*) to determine infiltration of the gel by neutrophils. *A*, A-5, weeks 3 and 6; A-5GM14, week 1. *B*, A-5G12b, weeks 2 and 6; A-5G12bRT1D, weeks 1 and 6. *C*, quantification of granulocyte recruitment.

Effect of G-CSF and GM-CSF for Tumor Growth and Progression *In vivo*. Additionally, we were able for the first time to establish the functional contribution of G-CSF and GM-CSF to tumor progression *in vivo* by demonstrating (*a*) enhanced tumor growth and invasion, (*b*) a stimulation of angiogenesis, and (*c*) an enhanced recruitment of inflammatory cells in heterologous tumor transplants of the growth factor-transfected cell clones.

(*a*) Factor-negative parental cells as well as GM-CSF transfectants formed benign cysts after subcutaneous injection. In contrast, subcutaneous injection of G-CSF transfectants produced invasive tumors with pronounced proliferation in the tumor periphery. This G-CSF–induced stimulation of tumor growth is in agreement with studies showing that the expression of G-CSF receptor in head and neck tumors and coexpression of G-CSF and its receptor in SCCs are associated with a higher rate of tumor recurrence and a worse prognosis for the patient (30, 37). In support of this, intraperitoneal application of G-CSF was shown to enhance the growth of subcutaneously injected tumors (38). Tumor growth in our system was further enhanced after *in vivo* passage of G-CSF transfectants, which was associated with a constitutive neoexpression of GM-CSF in these cells. We have first indications that the expression of GM-CSF might work its effect via an indirect mechanism by inducing the expression of IL-6, but not the expression of any of any of the well-known angiogenic factors such as vascular endothelial growth factor (VEGF) or platelet-derived growth factor.[2] This clearly supports the need for an additive or synergistic contribution of both factors to the establishment of an enhanced malignant tumor phenotype. Comparably, *de novo* expression of both growth factors has been reported in high-grade malignant gliomas, meningiomas, head and neck tumors, and SCCs of the skin (5, 8, 16, 17, 19, 20).

Remarkably, it is the *in vivo* tumor environment that exerts a characteristic selection pressure in favor of increasingly malignant tumor phenotypes associated with the coexpression of both growth factors (11). Such selection by the *in vivo* environment is supposedly induced by adverse environmental conditions, *e.g.,* hypoxia, low pH, and nutrient deprivation. In this context, a hypoxia-induced expression of IL-1 may initially contribute to an activation of G-CSF and GM-CSF expression in tumor cells (39). Eventually, that activation may become constitutive through the mutational influences that the hypoxic, low pH tumor stroma exerts on the tumor cells (40, 41).

(*b*) A critically important stromal contribution to rapid tumor growth is an enhanced angiogenesis, guaranteeing the supply of

[2] S. Schnur, N. Catone, E. Obermueller, W. Lederle, N. E. Fusenig, and M. M. Mueller. IL-6, a mediator of a growth factor network that promotes tumor progression in skin SCCs. Manuscript in preparation.

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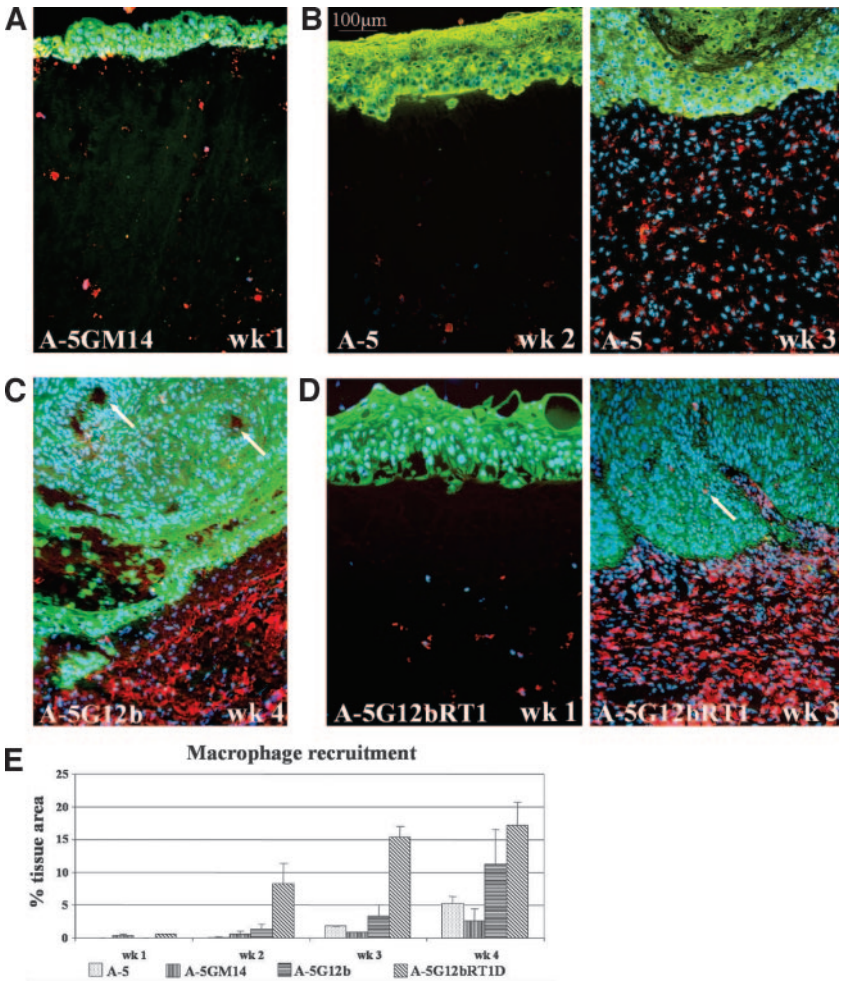

Fig. 9. Kinetics of macrophage recruitment. Cryosections of surface transplants were fixed with acetone and stained with antibodies against keratin (*green*), macrophages (*red*), and nuclei with Hoechst dye (*blue*) to determine infiltration of the gel by macrophages. *A*, A-5GM14, week 1. *B*, A-5, weeks 2 and 3. *C*, A-5G12b, week 4. *D*, A-5G12bRT1D, weeks 1 and 3 (*arrows* indicate macrophages infiltrating the tumor mass). *E*, quantification of macrophage recruitment.

nutrition and oxygen for the growing tumor (42). G-CSF expression and, in synergy with it, the co-expression of GM-CSF in the transfected cell lines induce an enhanced angiogenic response when compared with the parental A-5 cells and control transfectants. This was evidenced by persistent and strong angiogenesis throughout the observation period in transplants of the G-CSF transfectants. Additionally, *in vivo* progressed enhanced malignant A-5G12bRT cell lines with their coexpression of G-CSF and GM-CSF show a further enhancement of angiogenesis with an even stronger vessel recruitment than the G-CSF transfectants. Interestingly, as shown earlier for malignant HaCaT-ras tumor cells (43), persistent angiogenesis is a prerequisite for the development of an invasive tumor phenotype in the G-CSF transfectants and in the *in vivo* progressed A-5G12bRT1D cells as well. G-CSF and GM-CSF have been described to stimulate proliferation and migration of endothelial cells (12, 15) and to promote angiogenesis (14, 26, 44, 45), and the critical codependence between angiogenesis and invasion has been demonstrated previously by us and others (11, 43, 46). Thus, by stimulating angiogenesis, both factors clearly contribute to the efficient tumor vascularization and the resulting enhanced growth and invasion *in vivo*.

(*c*) An additional effect of G-CSF and GM-CSF in the stimulation of an activated and thus permissive tumor stroma lies in the stimulation of an enhanced leukocyte recruitment to the tumor-neighboring stroma and the effect of these factors on proliferation, maturation, and functional activity of granulocytes and macrophages. G-CSF and GM-CSF enhance the recruitment of granulocytes to the tumor site, which, for GM-CSF transfectants, is transient, as in the parental A-5 cells. In contrast to controls and GM-CSF transfectants, recruitment of granulocytes to G-CSF transfectants and the even further enhanced accumulation in the *in vivo* progressed G-CSF transfectant (A-5G12bRT1D), coexpressing both factors, were persistent throughout the observation period. This effect of G-CSF was further proven by transplantation of the factor in a collagen gel alone. This also induced an enhanced and persistent granulocyte recruitment into the granulation tissue formed as a result of the transplantation. Again, as seen for the G-CSF–transfected tumor cells, this granulocyte recruitment was accompanied by an enhanced and persistent angiogenic response in comparison with control transplants (data not shown).

Additionally, G-CSF and GM-CSF further modulate the tumor microenvironment through an enhanced and earlier recruitment of macrophages to the tumor vicinity, respectively. The role of macrophages for tumor growth and progression is a matter of controversial discussion. Although macrophages can mediate cytotoxicity and have been implicated in antitumor immunity (47, 48), tumor cells can develop mechanisms to escape and even benefit from the activities of the tumor-associated macrophages (49–51). In light of this potentially dual role, the early recruitment of macrophages in the GM-CSF transfectants, which reach the tumor already in week 1 after transplantation, might mediate a cytotoxic antitumor effect in these early stages of tumor growth. This could inhibit the tumor cell invasion in nude mouse surface transplantations *in vivo*, which was readily observed in organotypic skin equivalent cultures *in vitro* in which no macrophages were present. Many tumor-derived factors have been described to reduce

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the cytotoxic activity of tumor-associated macrophages (49, 51). Thus, the coexpression of G-CSF and GM-CSF might overcome the early antitumor activity, resulting in persistent angiogenesis and recruitment of granulocytes, and is therefore needed for the development of an enhanced malignant tumor phenotype. We are currently in the process of establishing an *in vitro* organotypic coculture system including these and other inflammatory cells to answer this question.

The critical importance of inflammatory cells as promoting forces in tumor development and progression has only recently been recognized (28, 49, 51). They are thought to contribute to tumor progression, on the one hand, by generating reactive oxygen and nitrogen species, which are responsible for combating infections, but also induce DNA damage in proliferating cells. Persistent and repeated exposure to these DNA-damaging agents can result in permanent genomic alterations that accumulate in previously normal cells and may thus initiate and promote malignant conversion (41). Additionally, tumor-associated inflammatory cells express a wide range of cytokines such as TNF-α, ILs, and interferons as well as angiogenic growth factors such as VEGF and basic fibroblast growth factor (51). In this context, granulocytes have been shown to alter the microenvironment via the release of soluble chemotactic factors and proteases (28). G-CSF induces the secretion of matrix metalloproteinase-9 in granulocytes and may thus contribute to the remodeling of the tumor stroma, allowing invasion and angiogenesis to occur (52). Furthermore, macrophages may stimulate tumor growth and angiogenesis by secreting cytokines and angiogenic factors, whereas macrophage-derived proteases degrade the surrounding tissue and thus facilitate tumor expansion, invasion, and angiogenesis (49–51, 53). Recent reports show that head and neck SCCs attract macrophages via Macrophage Chemotactic Protein-1 and transforming growth factor β1 and activate them to secrete angiogenic factors (basic fibroblast growth factor and VEGF) and the cytokines TNF-α and IL-1α, which then act in a paracrine fashion to stimulate tumor cells to produce IL-8 and VEGF, thus representing a double paracrine loop to induce angiogenesis (54). Thus, granulocytes and macrophages in the tumor environment have the ability to affect each phase of the angiogenic process, including degradation of the extracellular matrix and endothelial cell proliferation and migration (53), and can thereby either directly or indirectly promote tumor invasion, progression, and metastasis.

Collectively, our data demonstrate that coexpression of G-CSF and GM-CSF and their receptors in SCCs of the skin has multiple effects.

(*a*) G-CSF and GM-CSF function as part of an autocrine stimulatory loop directly enhancing tumor cell proliferation and migration. (*b*) They contribute to a more aggressive phenotype by stimulating tumor growth and invasion *in vivo*. (*c*) Both factors, although having clearly differential roles, act in a paracrine fashion on stromal fibroblasts, endothelial cells, and leukocytes, thereby generating a tumor microenvironment that promotes tumor growth and progression. Our data on the *in vivo* progression of the G-CSF transfectant to an enhanced malignant phenotype clearly demonstrate that coexpression of G-CSF and GM-CSF together has synergistic effects on tumor progression to a more malignant phenotype.

Questions concerning a decisive role of G-CSF and GM-CSF in the regulation of tumor growth and progression have become of clinical interest because both are now widely used adjuvants in routine cancer therapy protocols to control chemotherapy or radiation therapy-induced neutropenia. This therapeutic use relies on data showing no adverse effect of both factors (55). However, our data as well as that of other studies demonstrate the association of G-CSF and/or GM-CSF expression with an enhanced invasive and metastatic potential (5, 19) and thus warrant a careful reevaluation of the role of G-CSF and GM-CSF in the growth of solid tumors *in vivo* and the consequences of their use in cancer therapy protocols.

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