

# Granulocyte Colony-stimulating Factor Gene Transfer Suppresses Tumorigenicity of a Murine Adenocarcinoma In Vivo

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## Summary

We have investigated the effect of granulocyte colony-stimulating factor (G-CSF) delivery at the site of tumor growth by transducing, via retroviral vector, the human (hu) G-CSF gene into the colon adenocarcinoma C-26 and assaying the ability of transduced cells to form tumors when injected into syngeneic mice. As a control, the same tumor cells were infected with retroviruses engineered to transduce an unrelated gene, the human nerve growth factor receptor, or carry the neomycin resistance gene only. Only cells transduced with the huG-CSF were unable to develop tumors, although huG-CSF was expressed and produced at low level as estimated by both RNA analysis and enzyme-linked immunosorbent assay, indicating that G-CSF can exert an antitumor effect at a physiological dose. Implication of G-CSF as mediator of tumor inhibition was proven by reversing the nontumorigenic phenotype of G-CSF-expressing cells with anti-huG-CSF monoclonal antibody injected at the tumor site. No tumors were formed by injecting C-26 infected cells into *nu/nu* mice, while neoplastic nodules appeared after injection into sublethally irradiated mice; such tumors, however, regressed when mice normalized their leukocyte counts after irradiation. Tumors were also formed after injection of a mixture of infected and uninfected C-26 cells, although critical delay in tumor formation occurred when infected cells were 10 times more represented in the mixture. Histological examination of tissues surrounding the site of injection showed infiltration of neutrophilic granulocytes, whose number correlated with that of G-CSF-expressing C-26 cells in the injected mixture. These results indicate that G-CSF may have a potent antitumoral activity when released, even at low doses, at the tumor site. The antitumoral effect is mediated by recruitment and targeting of neutrophilic granulocytes to G-CSF-releasing cells.

Granulocyte colony-stimulating factor (G-CSF)<sup>1</sup> was first identified by its ability to induce differentiation of the murine myelomonocytic leukemia cell line WEHI-3B, and then was characterized as a potent differentiation-inducing CSF, essential in granulopoiesis (1). G-CSF shows a restricted action in stimulating progenitor hematopoietic cells in vitro, in that no eosinophil, erythroid, or megakaryocyte colonies are induced in clonal culture assays (1, 2). Human and mouse G-CSF bind equally well to receptors of either species and show complete biological crossreactivity (3). This allowed

for the study of the in vivo action of human G-CSF in mice, where it was shown to increase granulopoiesis in the spleen but also to stimulate erythropoiesis and megakaryocytopoiesis, and to increase the number of multipotential stem cells (CFU-S) (4, 5). Chronic exposure to the factor, studied in lethally irradiated mice reconstituted with syngeneic bone marrow infected with a retroviral vector expressing G-CSF cDNA, has been shown to induce non-neoplastic granulocytic and progenitor cell hyperplasia (6). Recombinant human (hu)G-CSF is now being tested in clinical trials for restoring granulocyte counts in myelodysplastic syndromes and for boosting granulocytic response after high-dose cancer chemotherapy (7-9).

Growing evidence indicates that several cytokines may have

<sup>1</sup> Abbreviations used in this paper: G-CSF, granulocyte colony-stimulating factor; hu, human; LAK, lymphokine-activated killer cell; LTR, long terminal repeat; Neo<sup>R</sup>, Neomycine phosphotransferase gene; NGF, nerve growth factor receptor.

*in vivo* antitumor activity, most effectively if they are delivered at the site of tumor growth (10–13). In fact, growth of different murine tumor cells engineered by gene transfer to produce IL-4, IL-2, or IFN- $\gamma$  is retarded or inhibited *in vivo* (11–13). IL-2 exerts its antitumor effect by eliciting a systemic immunity bypassing the requirement of a T helper function (12, 13), whereas the action of IFN- $\gamma$  is primarily mediated by upregulation of MHC class I gene expression (13). On the other hand, in the case of IL-4, the antitumor effect is apparently mediated by eosinophils and activated macrophages abundantly present at the site of tumor injection (11). Although it is unclear whether IL-4 elicits this response directly or by stimulating specific cytokine production by other cells, these studies suggest the possibility that phagocytic cells may effectively prevent tumor growth if appropriately targeted by cytokines.

We, therefore, focused our attention on the possible antitumor activity of G-CSF, which is known to act specifically both by stimulating the production of PMN neutrophilic granulocytes and by enhancing their migration to the cytokine production site. We used a retroviral vector to express huG-CSF in a poorly immunogenic murine colon adenocarcinoma, which was then injected in both conventional syngeneic and athymic mice to test the effect of G-CSF synthesis at the site of tumor-host interaction. We report here that tumorigenicity is virtually abolished in these cells through a mechanism that is independent of the T cell response and involves massive targeting of PMN granulocytes to the G-CSF-producing cells.

## Materials and Methods

**Tumors and Animal Studies.** C-26 is a murine colon adenocarcinoma induced in BALB/c mice by *N*-nitroso-*N*-methylurethan (14). C-26 tumor was maintained *in vivo* by subcutaneous transplant in syngeneic mice and adapted to culture in DMEM (Gibco Laboratories, Paisley, UK) supplemented with 10% FCS (Gibco Laboratories). BALB/c Ch and CD1 *nu/nu* mice were purchased from Charles River Breeding Laboratories (Calco, Italy) and maintained at the Istituto Nazionale Tumori by standard conditions, according to the Institutional Guidelines. Tumorigenic activity of control and virus-infected C-26 cells was assayed by injecting cells into the animal's right flank in a 0.2-ml volume via a 26-gauge needle of a 1-ml syringe.  $3 \times 10^4$  C-26 cells/mouse was the minimal dose required to kill 100% of injected mice. Higher doses were also used, as indicated in the tables and figures. The mixed tumor transplantation assay was performed by mixing control uninfected and virus-infected C-26 cells at 1:1 and 1:10 ratios ( $1 = 3 \times 10^4$ ) and injecting them into syngeneic mice as described above. Treated mice were scored for tumor growth twice a week. Experiments involving mAb administration were performed by using a mouse IgG1 anti-huG-CSF mAb (G-CSF, Ab-2; Oncogene Science, Manhasset, NY) and a mouse IgG anti-hu-NGFr mAb 20.4; (HB-8737; American Type Culture Collection, Rockville, MD) at concentration of 200 ng and 2  $\mu$ g/mouse/injection. mAbs were mixed with  $10^6$  infected (C-26/G3) and uninfected C-26 cells before injection into BALB/c mice; injections of the mAb around the site of tumor growth were repeated every 48 h for 10 d. The *in vitro* G-CSF neutralization activity of Ab-2 mAb is detectable at ratio of 100 ng of Ab to 100 pg of G-CSF (as reported by manufactory specifications), and the

doses injected *in vivo* theoretically represent 10 and 100 times the quantity of Ab required to neutralize the amount of G-CSF produced by G3 cells. Mice irradiation was performed by using a  $^{60}\text{Co}$  source ( $\sim 4,000$  Ci = 148,000 GBq; Theratron 780C, Atomic Energy of Canada Limited, Kanata, Ontario, Canada) to deliver a dose rate of 28 rad/min for a total dose of 600 rad. Treatment with anti-asialo GM $_1$  was performed using a rabbit antiserum (Wako Chemicals, Neuss, Germany). BALB/c mice were intravenously injected with 0.2 ml of diluted antibodies (0.5 mg) 3 d before tumor cell injection and 14 d later. Depletion of NK cells was assessed in the spleen of treated mice by a 4-h  $^{51}\text{Cr}$ -release assay against YAC-1 tumor target. BALB/c NK cells being weakly active, we performed the assay after depletion of adherent cells and boosting the NK activity by addition of rIL-2 (1,000 U/ml) during the 4 h of test incubation; in this way, we found abrogation of NK cytotoxicity (from 56% in spleen of untreated mice to no lysis in anti-asialo GM $_1$ -treated mice; E/T ratio: 100:1). In addition, we found abrogation of lymphokine-activated killer cell (LAK) production in spleens from anti-asialo GM $_1$ -treated mice by testing LAK cytotoxicity on P815 target cell after 3–5 d of *in vitro* activation with 500 U/ml of rIL-2. Leukocyte counts and blood formula were mechanically determined by an H1 apparatus (Technicon Chemicals Company, Tournay, Belgium).

**Retroviral Infection.** The NSV-G-CSF retroviral vector was constructed by cloning a 1.2-kb HincII/BalI cDNA fragment containing the complete coding region of human G-CSF (15) into the HindIII cloning site of the NSV vector. NSV is derived from the original N2 vector (16) by insertion of the 0.4-kb KpnI/HindIII fragment containing the SV40 early promoter and origin of replication into the unique XhoI cloning site downstream from the Neomycine phosphotransferase gene (Neo<sup>R</sup>). In this vector, the G-CSF cDNA is therefore under the control of the SV40 early promoter, whereas Neo<sup>R</sup>, used as a selectable marker, is driven by the Moloney murine leukemia virus long terminal repeat (LTR) (see Fig. 1B). The NSV-NGFr vector was constructed by inserting the 1.5-kb SstI cDNA fragment of the human nerve growth factor receptor (NGFr) (17) into the HindIII site of NSV. The LXS vector, containing Neo<sup>R</sup> under the control of the SV40 promoter, was previously described (18). All plasmid vectors were transfected into the  $\psi$ 2 ecotropic packaging cell line (19) by standard calcium phosphate co-precipitation (20). 48 h after transfection,  $\psi$ 2 supernatants were used to infect the amphotropic PA317 packaging line (21) for 12 h in the presence of 8  $\mu$ g/ml polybrene. Infected PA317 cells were selected in medium containing 0.8 mg/ml G418 (Gibco Laboratories), and then used to generate helper-free, virus-containing supernatants with titers ranging from  $10^4$  to  $5 \times 10^5$  CFU/ml.  $10^6$  C-26 target cells were infected for 4–12 h with undiluted supernatants containing 8  $\mu$ g/ml polybrene, grown for 48 h, and then selected in 0.5 mg/ml G418. Individual clones were isolated, expanded into cell lines, and injected into mice or subjected to further analysis.

**DNA and RNA Analysis.** High molecular weight DNAs were obtained from cells and tumors by standard phenol/chloroform extraction (20), digested to completion in 5- $\mu$ g aliquots with appropriate restriction enzymes, run on 0.8% agarose gel, transferred to nylon membranes (Hybond-N; Amersham International, Amersham, UK) by Southern capillary blotting (20), and hybridized to  $10^7$  dpm of DNA probe oligo-labeled to a specific activity of  $10^9$  dpm/ $\mu$ g, in the presence of 50% formamide and 10% dextran sulphate. Filters were washed at high stringency ( $0.2 \times$  SSC, 0.1% SDS at 55°C for 2 h) and exposed to Kodak X-AR5 films for 16–48 h at  $-70^\circ\text{C}$ . Total cellular RNA was extracted by the guanidine-isothiocyanate technique (22), run in 10- $\mu$ g aliquots on 1.0%

agarose-formaldehyde gel, transferred onto nylon membranes by Northern blot (23), and hybridized, washed, and exposed as described for Southern blots. DNA probes were the HincII/BalI fragment of huG-CSF cDNA (15) and a HindIII/SmaI fragment of pSV2-Neo (24) containing the Neo<sup>R</sup> gene.

**G-CSF Assay.** G-CSF production in cultured cells was quantitated by a human G-CSF quantitative ELISA from Oncogene Science (Manhasset, NY) and an EIA test kit from Amgen Biological (Thousand Oaks, CA). Cells were cultured at 10<sup>6</sup>/ml for 48 h, culture media were collected and concentrated 2× by ultrafiltration on a 30,000-mol wt cutoff membrane, and again concentrated 5× on a 10,000-mol wt cutoff membrane (Centricon; Amicon, Grace Italiana, Italy). Retenates from the 10,000 cutoff were tested according to manufacturer's instructions.

**Histologic Evaluation and Immunocytochemical Staining.** Tissues at the site of tumor cell inoculation were fixed in Bouin, blocked in paraffin, sectioned at 4 μm in triplicates, and stained with hematoxylin and eosin. For immunocytochemical staining, tissues were embedded in OCT compound (Ames Division, Miles Laboratories, Elkart, IN), snap frozen in liquid nitrogen, and stored at -80°C. Acetone-fixed 5-μm cryostat sections were blocked with rat serum and then immunostained with optimal dilution of the following rat mAbs: M1/9.3.4HL.2 (TIB 122) anti-CD45; 53.6.72 (TIB 105) anti-CD8; GK1.5 (TIB 207) anti-CD4; M3/84.6.34 (TIB 168) Mac3; and M1/70.15 (TIB 128) Mac1 were all obtained from the American Type Culture Collection as hybridoma cell lines. The slides were then sequentially incubated with goat anti-mouse Igs (Zymed Laboratories Inc., San Francisco, CA) and rat PAP antibodies (Abbott Laboratories, North Chicago, IL). Each incubation step lasted at least 30 min and was followed by a 10-mm PBS wash. Sections were then incubated with 0.03% H<sub>2</sub>O<sub>2</sub> and 0.06% 3,3'-diaminobenzidine (BDH Chemicals, Poole, England) for 2-5 min, washed in tap water, and counterstained in hematoxylin.

## Results

**Murine Colon Adenocarcinoma Cells Infected with NSV-G-CSF Lack Tumorigenicity In Vivo.** NSV-G-CSF viral supernatant produced during 24 h of semiconfluent culture of PA317 packaging cells was collected, titrated, and used to infect the C-26 murine colon adenocarcinoma. Eight independent G418-resistant C-26 colonies were isolated, grown, and tested for tumorigenicity in vivo by injection into syngeneic mice at a dose (3 × 10<sup>4</sup> cells/mouse) that produces tumors in 100% of animals injected with control C-26 cells. None of the NSV-G-CSF-infected colonies gave tumors in syngeneic mice (Table 1). Two colonies, named C-26/G3 and C-26/G4, were chosen for further studies. These cells showed no difference in both morphology and growth characteristics with respect to uninfected C-26 cells (data not shown). Tumorigenic activity of C-26/G3 and G4 cells was compared with that of uninfected C-26 cells and unrelated retrovirus-infected C-26 cells, including: (a) C-26 cells infected with the NSV-NGFr retroviral vector, in which the human NGF receptor cDNA replaced G-CSF in the same retroviral background; and (b) C-26 cells infected with the LXSN vector, containing the Neo<sup>R</sup> cassette under the SV-40 promoter (see Materials and Methods). The results (Table 2) indicate that among C-26 cells that acquired the G418 resistance phenotype by retroviral integration, only those that originated from the NSV-G-CSF infection were nontumorigenic.

**Table 1.** *In Vivo* Tumorigenicity of C-26 and C-26 NSV-G-CSF-infected Cells

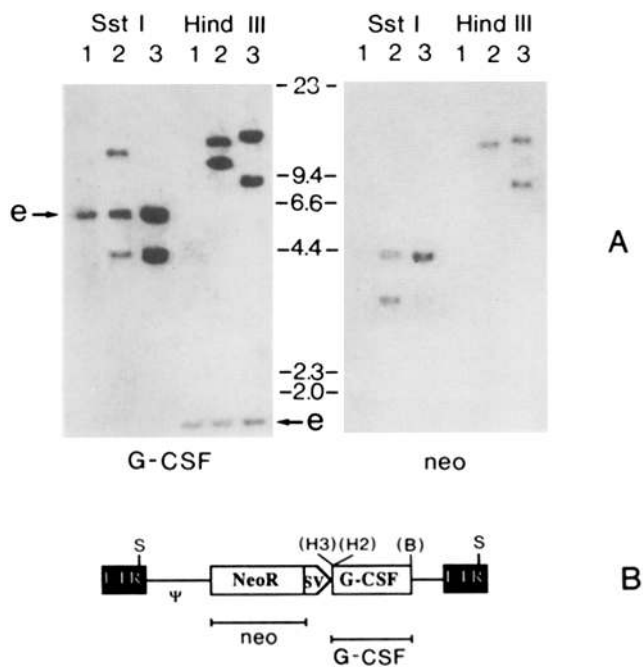
Uninfected C-26	NSV-G-CSF-infected C-26 colonies							
	1	2	3	4	5	6	7	8
10/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10

**Integration and Expression of HuG-CSF in C-26-infected Cells.** Integration of the NSV-G-CSF retroviral genomes was analyzed by Southern blotting of DNA from colonies C-26/G3 and G4. Fig. 1 A shows that a major 4.5-kb band, corresponding to the expected size for an intact integrated provirus, was observed in DNAs digested with SstI, which cuts once in both vector LTRs (Fig. 1 B), and hybridized to both huG-CSF and Neo probes. An additional high molecular weight band hybridizing with G-CSF but not the Neo probe was observed in the SstI digest from colony G3 (Fig. 1 A), indicating the presence of at least one rearranged proviral genome. A band corresponding to the crosshybridizing, endogenous murine G-CSF gene was observed in all samples hybridized to the huG-CSF probe. Digestion with HindIII, which does not cut in the provirus, gave rise to multiple bands in the DNA of both colonies, indicating the presence of different integration sites per haploid genome. Expression of vector-derived transcripts was analyzed by Northern blotting of total

**Table 2.** *In Vivo* Tumorigenicity of C-26 Cells Infected or Not with NSV-G-CSF and Different Retroviral Vectors and Selected with G418

C-26 infected with:	No. of mice with tumor/ No. of mice injected			
	3 × 10 <sup>4</sup> *	10 <sup>5</sup>	10 <sup>6</sup>	10 <sup>7</sup>
None	30/30	10/10	5/5	5/5
NSV-G-CSF colony 3 (G3)	0/30	0/20	0/20	0/10
NSV-G-CSF colony 4 (G4)	0/20	0/10	0/10	0/10
NSV-NGFr colony 3	5/5	ND	5/5	ND
NSV-NGFr colony 5	5/5	ND	5/5	ND
NSV	5/5	ND	5/5	ND
LXSN	5/5	ND	5/5	ND

\* No. of tumor cells injected.



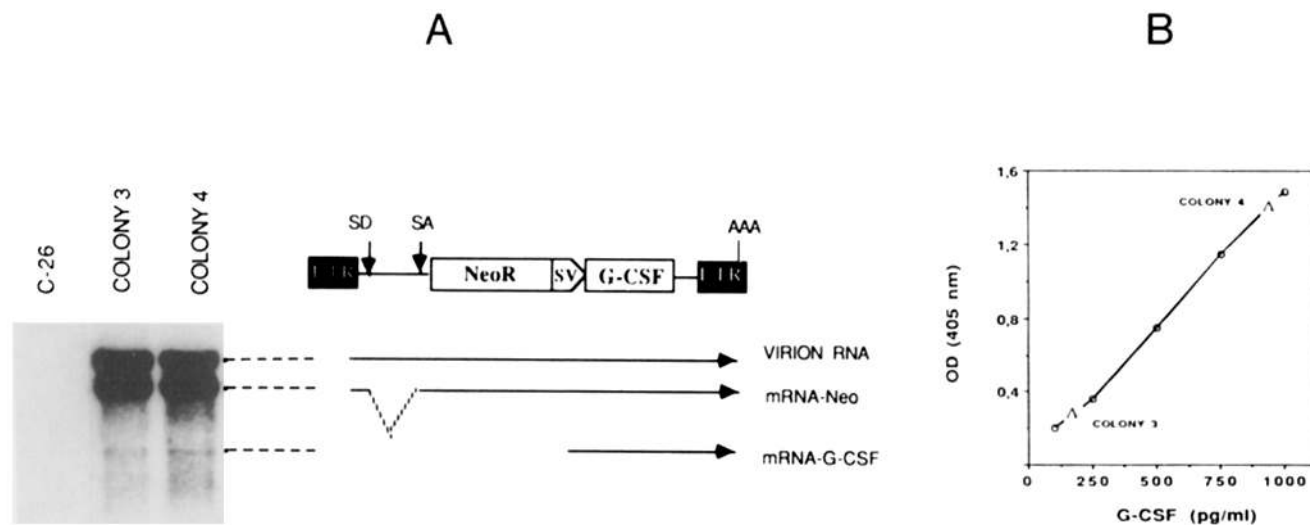
**Figure 1.** Analysis of NSV-G-CSF retroviral integration in C-26 cells. (A) DNA from uninfected C-26 (lane 1) and from G418 selected colonies G3 and G4 (lanes 2 and 3, respectively) were digested to completion with SstI or HindIII in order to perform Southern blotting. Hybridizations were sequentially performed with huG-CSF (left) and pSV2-Neo (right) probes; (e) indicates the endogenous G-CSF sequences. A  $\lambda$ -phage HindIII digest was used as size marker. (B) Schematic map of the NSV-G-CSF retroviral vector; SstI (A), HindIII (H3), HincII (H2), and Ball (B) restriction sites are indicated. Restriction sites indicated within parentheses were abolished during the NSV-G-CSF vector construction.

cellular RNA (Fig. 2 A). As expected for this type of vector (16), abundant unspliced and spliced 5'-LTR-generated transcripts were detected in RNA from both colonies, after hy-

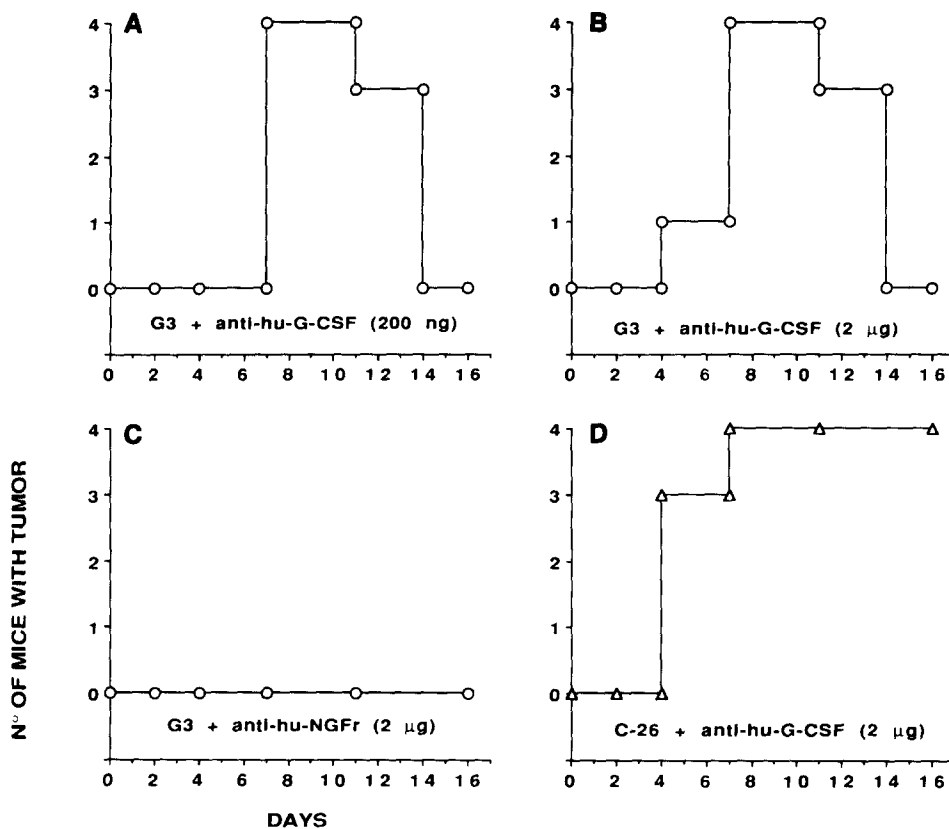
bridization with either G-CSF or Neo probes. A faint, 2.4-kb band hybridizing to the G-CSF probe was also detected, corresponding to the subgenomic, SV40-promoted G-CSF transcript. This indicates that activity of the internal SV40 promoter is low in murine C-26 cells.

Production of huG-CSF by infected C-26 cells was evaluated by immunoassay of the G-CSF levels in the culture medium. Conditioned media from a 48-h culture of  $10^6$  cells from colonies C-26/G3 and G4 and from uninfected C-26 cells were 10 times concentrated over a 30,000- and then a 10,000-mol wt cutoff, and assayed by two different commercial kits. Secretion of G-CSF from C-26/G4 and G3 amounted to 900 and 200 pg/ml, respectively, the latter value being at the edge of test sensitivity, while no G-CSF could be detected in the culture medium of uninfected C-26 (Fig. 2 B).

*Reversion of the C-26/G3 Nontumorigenicity by Anti-G-CSF mAb* To test directly the role of huG-CSF on C-26/G-3 tumor suppression,  $10^6$  G3 cells were injected with 200 ng or 2  $\mu$ g of anti-huG-CSF neutralizing antibody. Since the G-CSF produced by  $10^6$  G3 cells in 48 h was estimated to be 200 pg, mAb was given repeatedly every 48 h around the site of tumor injection. In this way, we estimated that we should deliver the amount of mAb that is 10 and 100 times its nominal in vitro blocking activity, respectively (see Materials and Methods). As a control, we injected G3 cells together with an unrelated mAb and the uninfected C-26 cells with anti-huG-CSF mAb. The results of this experiment are reported in Fig. 3 and indicate that G3 cells were able to form tumors (size ranging from 6 to 10 mm<sup>3</sup>) after treatment with anti-huG-CSF, but not with an anti-huNGFr mAb used as unrelated control. However, the antibody effect was time restricted and not limited to the treatment period since tumor regression began before the interruption of mAb injections. This should be explained either by a dilution of mAb per



**Figure 2.** Expression and production of huG-CSF in NSV-G-CSF-infected cells. (A) Northern blot analysis of total cellular RNA (10  $\mu$ g) from C-26 and C-26 NSV-G-CSF-infected colonies 3 and 4 (G3 and G4) and schematic representation of transcripts from the LTR and the SV-40 promoters, as detected by the huG-CSF probe. SD and SA indicate splice donor and splice acceptor sites, respectively. (B) ELISA determination, by the assay kit from Oncogene Science, of huG-CSF produced by colonies 3 and 4. ( $\Delta$ ) The average absorbance values, for both C-26/G3 and G4 cells, that intersect the standard curve prepared by plotting the average absorbance vs. the concentration of the G-CSF standard solutions.



**Figure 3.** Reversion of the C-26/G3 nontumorigenicity by anti-huG-CSF mAb. A murine anti-huG-CSF (A, B, and D) or anti-huG-NGFr (C) mAb were mixed to  $10^6$  G3 cells (A-C) or C-26 cells (D) before injection; mice were then injected peritumorally, at days 2, 4, 6, and 9 with the given mAb.

cell during tumor formation, or by an inefficient delivery of the mAb.

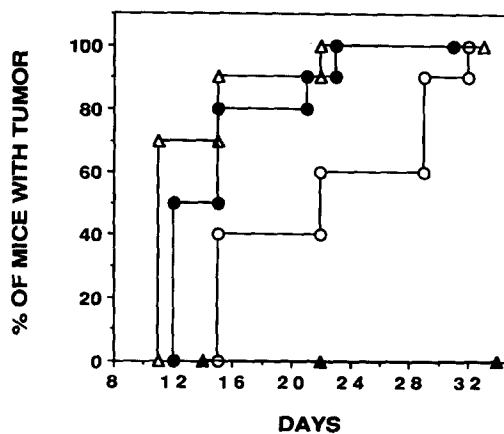
**Growth of C-26/G3 and G4 Cells in Immunologically Impaired Mice.** Although G-CSF shows a restricted activity on neutrophilic granulocytes *in vivo*, we deemed it interesting to know whether tumor suppression activity required the cooperation of other effector cells. To this aim, C-26/G3 and G4 cells were injected into: (a) athymic (*nu/nu*) mice; (b) mice depleted of NK cells by treatment with anti-asialo GM<sub>1</sub> antiserum; and (c) sublethally irradiated (600 rad) mice. The results showed that C-26/G3 and G4 cells were unable to develop tumors in *nu/nu* and NK-depleted mice (Table 3),

**Table 3.** Tumorigenicity of C-26 and NSV-G-CSF-infected C-26 Cells Injected into Immunologically Impaired Mice

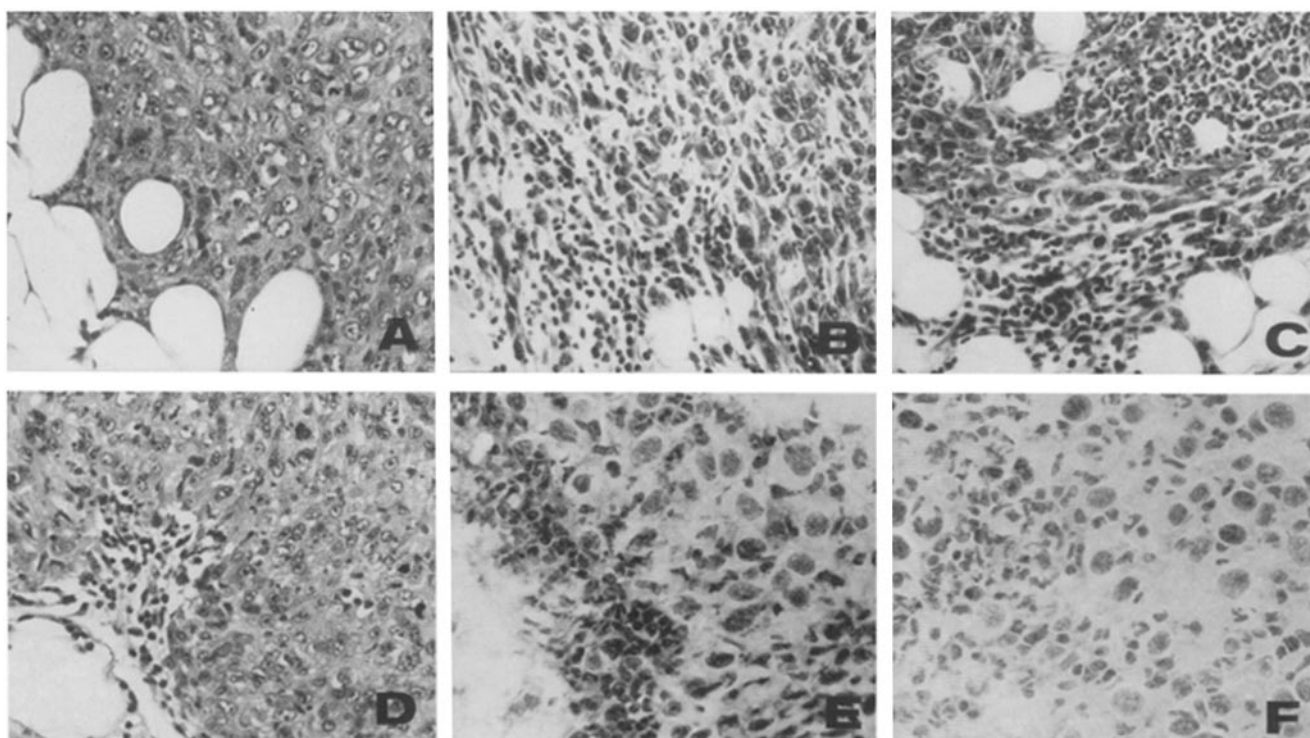
Cells injected*	No. of mice with tumor/No. of mice injected				
	BALB/c	BALB/c (600 rad)		BALB/c a-asialo GM1	<i>nu/nu</i>
		Day 30	Day 60		
C-26	5/5	5/5	5/5	5/5	5/5
C-26/G3	0/10	10/10	2/10	0/10	0/10
C-26/G4	0/10	10/10	1/10	0/10	0/10

\*  $10^6$  cells/mouse.

indicating that T and NK cells are not required for tumor suppression of G-CSF-producing cells. Conversely, transient tumor growth was consistently observed in irradiated mice: tumors grew to the size of  $\sim 1$  cm<sup>3</sup> followed by regression in 8 out of 10 mice injected with C-26/G3 and 9 out of 10 mice injected with C-26/G4 cells. Regressions were to completion, with reabsorption of tumors and resolution of even skin ulcerations when present. Follow-up of treated mice in-



**Figure 4.** A mixed tumor transplantation assay: tumor outgrowth after injection of the C-26/G4 and C-26 cell mixture. C-26/G4 cells ( $10^6$ ) were injected alone ( $\blacktriangle$ ) or mixed at a 1:1 ( $\bullet$ ) or 10:1 ( $\circ$ ) ratio with C-26 cells. ( $\triangle$ ) The C-26 cells injected alone.



**Figure 5.** Histological and immunocytochemical analysis of the site of tumor injection. Histopathology and immunoperoxidase staining of tumor sections from mice transplanted with uninfected C-26 (A), a mixture 1:1 (B) and 10:1 (C) of C-26/G4, or 1:1 (D–F) of C-26/G3 and uninfected C-26 cells. Intratumoral reactive cells showed mainly the morphologically or neutrophils, and their amount appears to be related to the number of infected cells present in the mixture (B vs. C) (A–D/hematoxylin-eosin;  $\times 400$ ). The neutrophilic origin of the reactive cells is supported by immunoperoxidase staining showing positivity for Mac1 (M1/70.15) (E) and negativity for Mac3 (M3/84.6.34) (F) (PAP method;  $\times 400$ ).

cluded a weekly determination of white blood cell counts and formulas, which indicated that normalization of leukocytes counts after the initial, radiation-induced reduction was necessary before any tumor regression became clinically appreciable. These data indicate that growth of G-CSF-producing tumors is no longer suppressed in the absence of granulocytes, and that mice self-reconstituted after sublethal irradiation are still able to destroy previously formed tumors.

**G-CSF-secreting C-26 Cells Do Not Suppress Tumorigenicity of Uninfected C-26 Cells in a Mixed Tumor Transplantation Assay.** Additional insight into the mechanisms by which G-CSF production causes inhibition of tumor growth in vivo came from mixed tumor transplantation experiments in which 1:1 and 10:1 mixtures of C-26/G4 and uninfected C-26 cells were injected in syngeneic mice. Tumors arose in 100% of the animals, although a longer latency was scored with the 10:1 ratio mixture (Fig. 4). After the injection of the 1:1 mixture of C-26/G3 and C-26 cells, tumors developed in all the treated animals as well as the G4 plus C-26 mixture (data not shown). Southern blot analysis of DNA extracted from these tumors failed to detect the presence of NSV-G-CSF sequences (data not shown), suggesting that they originated from uninfected C-26 cells. These results indicate that inhibition of tumor cell growth by G-CSF is only occurring in cells actually secreting the hemopoietin. This conclusion was corroborated by experiments showing that recombinant huG-CSF injected together with C-26 cells, and subsequently given

around the injection site twice a day for 20 d at a dose of  $400 \mu\text{g/day}$ , was unable to inhibit C-26 tumor formation (data not shown).

**Histological and Immunocytochemical Analysis of the Site of Tumor Injection.** To elucidate which host cells were activated and elicited by G-CSF-producing cells in vivo, morphological and immunocytochemical analysis of the reactive populations present at the tumor site was performed 5 and 10 d after injection of C-26, C-26/G3, C-26/G4, and of their 1:1 or 1:10 mixtures. C-26 tumor showed a solid growth, with central necrotic areas. Intratumoral reactive cells were absent, and only a few granulocytes and macrophages were observed in the fat tissue around the tumor (Fig. 5 A). In mice injected with G3 and G4 cells, tissues surrounding the site of injection did not show any histological alterations. Compared with C-26, tumors arising after mixture inoculations were of smaller size and devoid of necrotic foci. Furthermore, they were characterized by a prominent intratumoral reactive infiltrate, the amount of which was related to the number of G-CSF-producing cells present in the mixed population (Fig. 5, B and C). The reactive cells were positive to Mac1 staining and negative for both Mac3 (Fig. 5, E and F) and a mixture of anti-CD4 and anti-CD8 mAb (data not shown). Mac1 stains granulocytes and macrophages while Mac3 only stains macrophages. The restricted positivity for Mac1 and morphological features identified the reactive cells as PMN neutrophilic granulocytes. Presence of eosinophils was ruled

out by the absence of spontaneous peroxidase in control and Mac3- or CD4/CD8-stained slides.

## Discussion

The study of the effect of lymphokines directly released at the site of tumor host interaction has been made possible by transfection procedures that allow interleukin genes to be expressed in tumor cells injected into mice (11–13). In this study, we have used a retroviral mediated gene transfer to transduce the human G-CSF gene into the murine colon adenocarcinoma C-26 to study how G-CSF-producing C-26 cells behave in vivo. Our results show that only C-26 cells that transduced the human-G-CSF gene lacked tumorigenicity (even at the dose of  $10^7$  cells/mouse), whereas uninfected C-26 cells and cells infected by the same retroviral vector, either empty or carrying a control gene, caused tumors when injected at the dose of  $3 \times 10^4$  cells/mouse. The effect of huG-CSF in this tumor was remarkable considering its low level of expression. This is a normal drawback of the NSV-derived vectors, which although they generate high-titer viruses, often result in low expression of the internal, SV40-promoted gene (25). This boosts the evidence that G-CSF can exert an antitumoral effect at a concentration possibly close to the physiological one, and suggests that a local, microenvironmental release of G-CSF may be more active than a large and systemic availability. Indeed, the tumor suppression activity of G-CSF was limited to the G-CSF-producing cells and not transferred to the nonproducing C-26 cells in a mixed tumor transplantation assay (Fig. 4). We hypothesize that a gradient of G-CSF concentration around G-CSF-producing C-26/G3 and G4 cells is instrumental in targeting effector cells, thus causing the destruction of G3 and G4 cells over that of C-26 cells. C-26 outgrowth was only delayed when G4 were 10 times more represented than C-26 cells in the injected mixture. It has been described that cell surface contact or cell-cell interactions are essential to trigger the respiratory burst of PMN activated by soluble stimuli (26). Likewise, an intimate contact between effector and G-CSF-producing cells is strictly necessary, since we have found that G3 or G4 cells segregated by millipore filters into a diffusion chamber placed subcutaneously in syngeneic mice were not destroyed (data not shown). The implication of G-CSF as the mediator of tumor inhibition was strengthened by experiments showing that a mouse anti-huG-CSF mAb was able to reverse the nontumorigenic phenotype of infected C-26/G3 cells (Fig. 3); such reversion was, however, time restricted, suggesting that the mAb needs to be present in the microenvironment surrounding the G-CSF-producing cells in order to exert an efficient blocking activity.

Although the G-CSF have a restricted activity on neutrophilic granulocytes, we investigated whether tumor suppression activity required the cooperation of other host cells by injecting C-26/G3 and G4 cells into mice immunologically deficient for either the T or the NK lineage. The G-CSF-producing cells did not develop into tumors when injected into *nu/nu* mice, but they did when given to irradiated (600 rad) BALB/c mice, indicating an immunological

non-T-mediated mechanism of tumor destruction. Implication of NK cells was ruled out by treating mice with anti-asialo GM<sub>1</sub> antibodies, by the known NK radioresistance and by the poor lysability of this tumor by NK cells. Since G-CSF has a restricted activity on neutrophilic granulocytes, we assumed that this population was the most involved at the site of tumor growth. Eosinophilic granulocytes, macrophages, and T lymphocytes were previously reported to be stimulated by IL-4 or peritumoral injection of IL-2 (11, 27). To document which cells were elicited and activated at the site of tumor injection, all tissues surrounding the point of injection of the G3 or G4 plus C-26 mixtures were histologically examined. The results indicated that neutrophilic granulocytes were the only cells infiltrating the mixture and that the extent of PMN infiltration correlated with the number of G-CSF-producing cells injected.

The finding of antitumoral effect of neutrophilic granulocytes stimulated by tumor-released G-CSF is intriguing in the light of the known ability of some tumors to induce neutrophilia and to secrete CSF. In the case of murine mammary carcinoma CE, neutrophilia was due to the tumor-derived G-CSF and, in vitro, granulocyte stimulation was enhanced by the synergistic action of G-CSF and purified M-CSF (28) but no antitumoral activity was found. This can suggest that, in our system, some other factor(s) released by C-26 may cooperate with the G-CSF in suppressing tumor growth in vivo. Interestingly, C-26 induces cachexia and neutrophilia, as found by following the blood formula during the time of tumor growth, the onset of neutrophilia being associated with at least 1 cm<sup>3</sup> of tumor size. However, we could not detect endogenous G-CSF expression in C-26 cells by Northern blot using the crossreactive human probe. Mice injected with G3 cells never developed tumor and never deviated from the normal blood formula. Altogether these observations would support the requirement of additional factors concurring with G-CSF to exert the observed potent antitumoral effect. Furthermore, it seems likely that different tumors behave differently when attacked by neutrophilic granulocytes. In this respect, in vitro studies revealed that depending on the target cell type and the condition under which the interaction occurs, the PMNs may disrupt a monolayer architecture with or without cell killing (29). Insensitivity to PMN-mediated killing may explain why, even in the presence of large numbers of PMN, the tumor grows progressively. In addition, different tumor cells display a different susceptibility to the same cytokine, as exemplified by the fact that forced expression of IL-2 gene into C-26 colon adenocarcinoma can stimulate host T cell response (12), whereas IL-2 transduction by retroviral vector into the CTLL-2 lymphoma is associated with the fatal outcome of the injected mice (30). Recombinant huG-CSF was already described as being able to stimulate both differentiation and self-renewal processes of murine leukemic stem cells in vitro, but also of suppressing leukemia development in vivo, probably through the cytotoxic action of PMN (31). However, the mechanisms by which G-CSF primes neutrophils for enhanced oxidative metabolism (32), antibody-dependent cell-mediated cytotoxicity (33), and arachidonic acid release (34) remain to be elucidated.

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