American Journal of Pathology, Vol. 150, No. 3, March 1997

Ectopic G-CSF Expression in Human Melanoma Lines Marks a *Trans*-Dominant Pathway of Tumor Progression

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Using a human melanoma/Scid xenograft model with the C8161, M24-met, LD-1 and other human melanoma lines to investigate spontaneous metastasis, we made the observation of marked splenomegaly (up to five times normal weight and size) in only those xenografts exhibiting high degrees of spontaneous metastasis. Evaluation of this revealed the cause to be massive myelopoiesis due to ectopic granulocyte/colony-stimulating factor (G-CSF) production by the melanoma cells. Because of these observations linking G-CSF expression with metastasis of human melanoma, we decided to investigate the mechanism of this ectopic production. No gross amplification or rearrangement of the G-CSF gene could be detected as the basis for the increased transcriptional activity in any of these lines. Human-human somatic cell hybridization studies carried out between the metastatic C8161 and several different non-metastatic non-G-CSF-expressing lines revealed, in addition to metastatic dominance, 3- to 10-fold enhancement of G-CSF transcription and expression in the fusions compared with C8161 itself. The suggestion of a trans-dominant mechanism was further supported by transfection studies with a human G-CSF promoter-CAT-reporter construct, which revealed 3- to 5-fold increased reporter activity in only those melanoma lines and hybrids expressing G-CSF. Furthermore, no obvious autocrine or paracrine effects of this ectopic G-CSF expression on the melanoma lines' growth or metastasis were apparent, as all of the G-CSF-expressing lines lacked the G-CSF receptor and injections of purified recombinant G-CSF exerted no stimulatory effects on their tumorigenicity, latency, growth, or metastasis in Scid mice. Thus, we advance the hypothesis that G-CSF expression is serving as a marker of a more generalized trans-dominant pathway linked to tumor progression and metastasis. This hypothesis has direct relevance to many human cancers where ectopic hormone or growth factor production occurs with no obvious autocrine or paracrine benefit to the tumor. (Am J Pathol 1997, 150:949–962)

The ability of human cancers to express genes that are not expressed by their normal cellular counterparts was first recognized several decades ago clinically as the phenomenon of ectopic hormone production.[1] It is still not clear in most instances whether the phenomenon of ectopic hormone production is a mere stochastic event linked to genetic instability or a selective event linked to either autocrine, paracrine, or some, as yet undefined, stimulation of tumor cell growth. The consistent association of a given ectopic hormone with a given tumor type, eg, small-cell carcinoma and antidiuretic hormone,[2] renal cell carcinoma and erythropoietin,[3] and squamous cell carcinoma and parathyroid hormone-related protein[4] suggests that the phenomenon is not stochastic. The lack of the corresponding receptor on small-

This work appeared in platform presentation at the American Association of Pathology Experimental Biology 1994 Meetings.

Supported by grants from United States Public Health Service (CA 40225 and CA 01351) and funds from the California Institute for Cancer Research, the Cancer Research Coordinating Committee, the Jonsson Comprehensive Cancer Center, and the Cancer Center core grant CA-16042. S. Safarians is a predoctoral trainee supported by the Mangasar Mangasarian Foundation. S. H. Barsky is a recipient of a National Institutes of Health Research Career Development Award.

Accepted for publication November 14, 1996.

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cell, renal cell, and squamous cell carcinoma cell lines in most instances and the lack of a growth response of these cell lines to exogenous hormone,[5,6] however, suggest that autocrine stimulation is not occurring. Possible paracrine benefits to the tumor are also not obvious.

Ectopic granulocyte/colony-stimulating factor (G-CSF) expression is an example of ectopic hormone production noted previously both in clinical melanoma and in melanoma cell lines.[7,8] Our laboratory in initially studying spontaneous metastasis with the human melanoma C8161 line[9,10] noted marked splenomegaly in the mice harboring the largest tumors. We found the cause to be ectopic G-CSF production by the tumor cells causing myelopoiesis. We then investigated this phenomenon in a number of different human melanoma lines and observed a strong correlation between ectopic G-CSF expression and metastatic ability. Because the mechanism of ectopic G-CSF expression in melanoma had not been previously addressed and because ectopic G-CSF could be serving as either an autocrine or paracrine factor in this setting promoting tumor cell growth and/or metastasis, we decided to investigate these issues in the present study.

Materials and Methods

Cell Lines

The study utilized the highly metastatic human amelanotic melanoma line C8161[11] (Dr. Mary J. C. Hendrix, University of Arizona, Arizona Cancer Center, Tucson, AZ). Other human melanoma lines used in this study included C8146a[12] (Dr. Frank Meyskens, University of California Clinical Cancer Center, Irvine, CA), BRO[13] (Dr. Beppino Giovanella, St. Joseph Hospital Laboratory for Cancer Research, Houston, TX) SK-MEL-28, A375, RPMI-7951[14–17] (American Type Culture Collection, Rockville, MD), M15 and M24[18] (Dr. Reiko F. Irie, John Wayne Cancer Center, Santa Monica, CA), a metastatic variant of the latter line, M24-met[19] (Dr. Ralph Reisfeld, Scripps Research Institute, La Jolla, CA), and a human melanoma line, LD-1, previously reported to secrete a factor that stimulated primarily granulocytic colonies in human and murine bone marrow cultures[8] and derived from a patient exhibiting marked granulocytosis (Dr. Mansoor Saleh, University of Alabama at Birmingham, Birmingham, AL). Other human nonmelanoma lines used in the study included a control cell line for the G-CSF receptor, HL-60[20] (Dr. Gayle Baldwin, Department of Medicine, Hematology, and Oncology, University of California, Los Angeles), and an immortalized nontumorigenic human matrix-secreting myoepithelial line, HMS-1.[21,22]

Somatic Cell Hybridizations

The study fused the metastatic C8161 line to several different nonmetastatic non-G-CSF-expressing human lines. A total of 10^5 cells from each cell line plated in 100-mm tissue culture dishes (Becton Dickinson Labware, Lincoln Park, NJ) were grown to 50% confluency in minimal essential medium (MEM) with Earle's salts supplemented with 10% fetal calf serum (FCS; Gibco BRL, Grand Island, NY). Each cell line was separately transfected with psv2*neo* (American Type Culture Collection) and p*Hyg* (Dr. Bill Sugden, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI). Transfections were carried out using Lipofectin reagent (Gibco BRL) according to the manufacturer's protocol. Cells were fused as described previously[23,24] with modifications. Briefly, 10^6 cells of each parental line were mixed and plated into 60-mm culture dishes (Corning, Corning, NY) with MEM plus 10% FCS and incubated overnight at 37°C in 5% CO_2/95% air. In some fusions, unequal numbers of parental cells were mixed to optimize successful hybridization. After pretreatment of cells with dimethylsulfoxide or glycerol, 3 ml of 50% (w/v) polyethylene glycol ($M_r \approx 1000$) (Fluka, Buchs, Switzerland) were added onto the dish and incubated at room temperature for 1 to 2 minutes. After removing the polyethylene glycol, the cells were washed three times with basal MEM (no FCS) and incubated in growth medium for 24 hours at 37°C in 5% CO_2/95% air. The cells were then harvested with trypsin/EDTA (Sigma), suspended in MEM plus 10% FCS, and plated into 100-mm tissue culture dishes at 8×10^5 cells in growth medium containing both 0.4 to 0.6 mg/ml G418 and 0.05 to 0.1 mg/ml hygromycin B. Emerging clones were examined by flow cytometric and chromosomal analysis.[25,26] Based predominantly on the modal numbers and ranges as well as the presence of marker chromosomes of each of the parents and their fusion products, it was determined which clones produced by somatic cell fusion were complete and stable. By this analysis, only clones exhibiting full and stable fusion were selected for subsequent studies.

G-CSF and G-CSF Receptor Studies

Enzyme-Linked Immunosorbent Assay (ELISA) for G-CSF

An ELISA for G-CSF (R&D Systems, Minneapolis, MN) was used to measure G-CSF in conditioned media, concentrated with Centriprep-10 concentrator (Amicon, Beverly, MA) from the tumor cell lines. The sensitivity of this G-CSF ELISA was 4.7×10^{-7} pg/cell.

G-CSF Southern and Northern Blots

Plasmid DNA was isolated using Qiagen plasmid kit (Qiagen, Chatsworth, CA), according to the manufacturer's directions. Probes were prepared by liberating fragments of interest from plasmids and gel purifying them using an electroeluter (International Biotechnologies, New Haven, CT). Labeling was accomplished by random prime labeling (Multiprime DNA labeling system, Amersham, Arlington Heights, IL) of 25 ng of probe with [α-^{32}P]dCTP (ICN Pharmaceuticals, Irvine, CA) at a specific activity of $\geq 5.0 \times 10^9$ cpm/μg. Sephadex TE Select-D G-50 columns (5 Prime-3 Prime, Boulder, CO) were used to remove unincorporated nucleotides. The pG-CSF6 plasmid[27] containing a 700-bp human G-CSF cDNA (Dr. David Tweardy, Pittsburgh Cancer Institute, Pittsburgh, PA) and the pGCAT plasmid[28] containing a 330-bp human G-CSF promoter fragment (−311 to +18; Dr. M. Frances Shannon, Institute of Medical and Veterinary Science, Adelaide, South Australia) were used. High-molecular-weight DNA was extracted as described[29] from confluent cultures and digested separately with *Eco*RI, *Bgl*II, and *Hin*dIII. After fractionating digested DNA on 0.8% agarose gel and transferring to nylon membranes, hybridizations were performed with QuikHyb solution (Stratagene, La Jolla, CA) according to the manufacturer's directions. After stringency washes, the membranes were exposed to Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY) with intensifying screens at −80°C. RNA was isolated by lysis of confluent cultures with the ULTRASPEC RNA isolation system (Biotecx, Houston, TX) according to the manufacturer's directions. Poly $(A)^+$ mRNA was isolated from total RNA using an oligo(dT) column (5 Prime-3 Prime) and dissolved in diethyl-pyrocarbonate-treated water. Both total and poly $(A)^+$ RNA were quantified spectrophotometrically, and 5 to 10 μg of poly $(A)^+$ mRNA was fractionated on a 1% agarose-formaldehyde gel and transferred to nylon membranes. Hybridizations in QuikHyb and exposure to film were performed as in DNA analysis. Normalization was with a β-actin probe.

G-CSF Receptor Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR for the G-CSF receptor was performed with a kit (Clontech, Palo Alto, CA) using 5′ primer 5′AAGAGCCCCCTTACCCACTACACCATCTT3′ and 3′ primer 5′TGCTGTGAGCTGGGTCTGGGACACT-T3′. First-strand cDNA was synthesized from total RNA template using a first-strand synthesis kit from 5 Prime-3 Prime. Briefly, to 24, 2.4, 0.024, and 0.0024 μg of total RNA from each cell line, 0.5 U of Inhibit-ACE (RNAse Inhibitor) and 0.5 μg of oligo(dt) primer were added. Synthesis of first-strand cDNA was performed in 20 μl total volume with 0.5 U of Inhibit-ACE, first-strand buffer, 10 mmol/L dithiothreitol, 0.5 mmol/L each of dATP, dCTP, dGTP, and dTTP, and 200 U of murine Moloney leukemia virus reverse transcriptase at 42°C for 1 hour. RT-PCR was carried out using 2 μl of each cDNA sample and 48 μl of master mix containing PCR buffer (50 mmol/L KCl, 10 mmol/L Tris/HCl, pH 8.3), 6 mmol/L MgCl, 0.2 mmol/L each of dATP, dCTP, dGTP, and dTTP, 0.4 μmol/L each of the 5′ and 3′ primer, and 2 U Ampli-*Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT). Total RNA template extracted from HL-60 served as positive control.

G-CSF Promoter Studies

CAT and β-galactosidase reporter plasmids were transfected into a number of different non-G-CSF-expressing and G-CSF-expressing cell lines and their somatic cell hybrids. Twenty micrograms of pGCAT and pβgal-basic (Clontech) were co-transfected into the different cell lines by using Lipofectin reagent (Gibco BRL) according to the manufacturer's protocol. pSV2CAT and pBL3CAT were used, respectively, as positive and negative (baseline) CAT reporter controls. At 72 hours after transfection, cells were lysed and extracts were analyzed for CAT reporter activity using a sandwich ELISA kit (Boehringer-Mannheim, Mannheim, Germany) that measured CAT activity (nanograms per microgram of cellular protein). Values obtained were normalized for transfection efficiency by measurements of β-galactosidase using a luminescent β-galactosidase detection kit (Clontech). Transfection efficiencies varied at most 2.5-fold in the various melanoma lines examined.

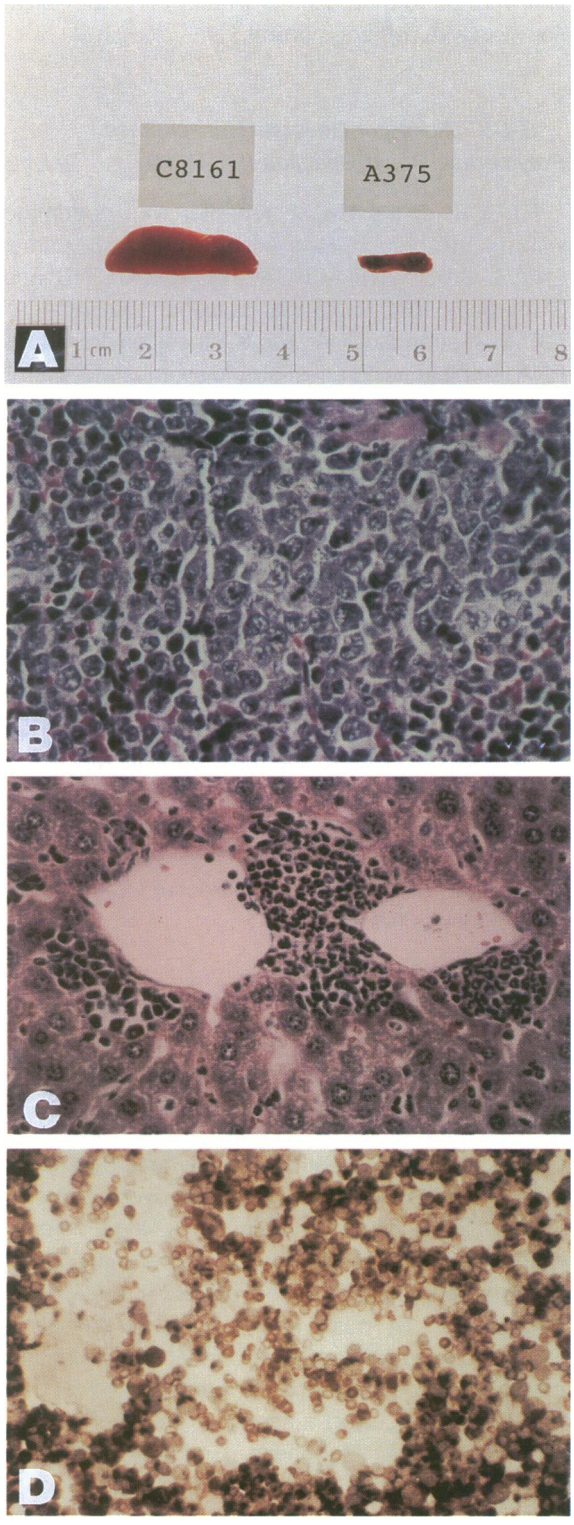

Figure 1. A: *Contrasting sizes of spleens from C8161-bearing Scid mice* (left) versus *A375-bearing mice* (right). *Splenomegaly was present up to five times normal in the C8161-bearing mice and was directly proportional to tumor burden.* B: *Microscopic sections of spleen revealed the cause of the splenomegaly to be massive myelopoiesis. Sheets of immature myeloid cells are seen within splenic parenchyma. The appearance of bone marrow was identical.* C: *Sections of liver showed focal collections of myelopoiesis consisting of both immature and mature myeloid elements (granulocytes).* D: *An air-dried touch prep of the spleen stained with Sudan black B depicts large numbers of positive-staining myeloid cells. Similar results were demonstrable with myeloperoxidase stains.*

Other Molecular and Immunocytochemical Studies

Other probes used in this study included *nm23* cDNA probe[30] (Dr. Patricia Steeg, National Institutes of Health, Bethesda, MD) and human DNA methyltransferase cDNA[31] (Dr. Paula Vertino, Johns Hopkins Oncology Center, Baltimore, MD). Murine monoclonal antibodies used to demonstrate differentiation status of the different melanoma lines as well as the fusions included antibodies to cytokeratins 8 and 18[32] and melanoma-associated antigens including S100 protein, HMB-45, and NKI/C3[33–35] (Abbot Laboratories, Chicago, IL). Monoclonal antibodies used to investigate potential cellular pathways involved in the *trans*-dominant acquisition of G-CSF expression included p53-Ab2[36,37] (Oncogene Sciences, Uniondale, NY) and EGFR-Ab[19] (CIBA-Corning, Medfield, MA). The second antibody was an affinity-purified peroxidase-conjugated sheep anti-mouse IgG. Immunoreactivity was measured by established methods.

Tumorigenicity and Metastasis Studies

Four-week-old male Scid mice were used. From 10^4 to 10^6 cells suspended in 0.2 ml of basal MEM (no FCS) were injected subcutaneously in the right dorsolateral flank region; tumors arose within 2 to 4 weeks and were allowed to grow to various sizes (0.5 to 2.5 cm in diameter) over the ensuing 3 to 7 weeks or until the animals became moribund. At selected times, animals were euthanized and autopsied. Metastases were detected by histological analysis of the lungs and by retrieval of pulmonary colonies through collagenase digestion, antibiotic selection, and clonal outgrowth. Other organs were removed and studied grossly and histologically. Specifically, the sizes and weights of the spleens were recorded. Before sacrifice, mice were bled from the tail vein and studies of peripheral blood were performed by Coulter counter (Coulter Electronics, Marietta, GA) and blood smear analysis. Specifically, the white blood cell count with differential, hematocrit, and platelet count were determined. The above experiments were repeated in mice receiving injections of human recombinant G-CSF (rG-CSF; R&D Systems). Select groups of mice were subcutaneously injected with human rG-CSF at a dose of 2.5 μg/injection twice daily for 1 week before receiving an injection of tumor cells. At the time of tumor cell injection, neu-

trophil counts were monitored. The injections of rG-CSF were continued over the ensuing 4 to 7 weeks. Tumorigenicity, tumor growth, and spontaneous metastasis were observed for the different melanoma lines being studied. Control groups received injections of Hanks' balanced salt solution. All experiments were performed with groups of 10 mice. Results were analyzed with standard tests of statistical significance, including a two-tailed Students' *t*-test, and a one-way analysis of variance.

Results

Marked splenomegaly was in evidence in mice harboring the C8161, LD-1, and M24-met xenografts especially when the xenografts were >2.0 cm in size (Figure 1A). At this point, widespread metastases were also usually evident. Studies performed postmortem on histological sectioning of the organs revealed marked granulocytosis and myelopoiesis (Figure 1, B–D). The myelopoiesis was seen involving the spleen, liver, and bone marrow. The myelopoiesis in the bone marrow was so marked that erythroid and megakaryocytic elements were markedly decreased; the liver exhibited foci of extramedullary myelopoiesis, and the spleen contained marked myelopoiesis with decreased erythroid elements but an apparent compensatory increase in megakaryocytes. Antemortem peripheral blood counts showed a markedly elevated white blood cell count to 125,000/mm^3 (normal Scid, 8,000/mm^3) composed exclusively of mature granulocytes. The overall degree of myelopoiesis, splenomegaly, and granulocytosis correlated with size of primary tumor and overall tumor burden (Figure 2, A and B). The other human melanoma lines examined, including C8146a, BRO, SKMEL, A375, RPMI, M24, and M15, showed no effects on myelopoiesis. Because human G-CSF was one of only a very few human hematopoietic growth factors that could cross target murine hematopoietic cells[38] and because it had been reported before in human melanoma,[7,8] it was considered a prime candidate for the observed effects on myelopoiesis. Using a G-CSF ELISA, markedly elevated levels were detected both in C8161-, LD-1-, and M24-met-conditioned media (up to 2 × 10^{-3} pg/cell) as well as mouse serum from Scid mice harboring these melanoma xenografts. None of the other melanoma lines, including M24, nor the myoepithelial line HMS-1 revealed any detectable G-CSF.

Southern blots of the C8161, LD-1, and M24 met lines compared with blots of the the non-G-CSF-expressing melanoma lines and HMS-1 probed with the 700-bp G-CSF cDNA revealed no evidence of gross gene amplification or rearrangement as the mechanism of altered expression in the G-CSF-expressing lines (Figure 3A). Probing with the 330-bp G-CSF promoter probe also revealed no evidence of gross gene amplification or rearrangement. Comparative Northern blots of C8161, LD-1, and M24-met with the non-G-CSF-expressing cell lines revealed a prominent 1.6-kb G-CSF transcript in only the G-CSF-expressing lines, indicating that the regulation of G-CSF expression was transcriptional (Figure 3, B and C). As the basis of the increased G-CSF expression was increased transcription, yet no obvious G-CSF structural gene alterations were seen, we decided initially to carry out somatic cell hybridizations between C8161 and the many different non-G-CSF-expressing cell lines, including HMS-1, to investigate

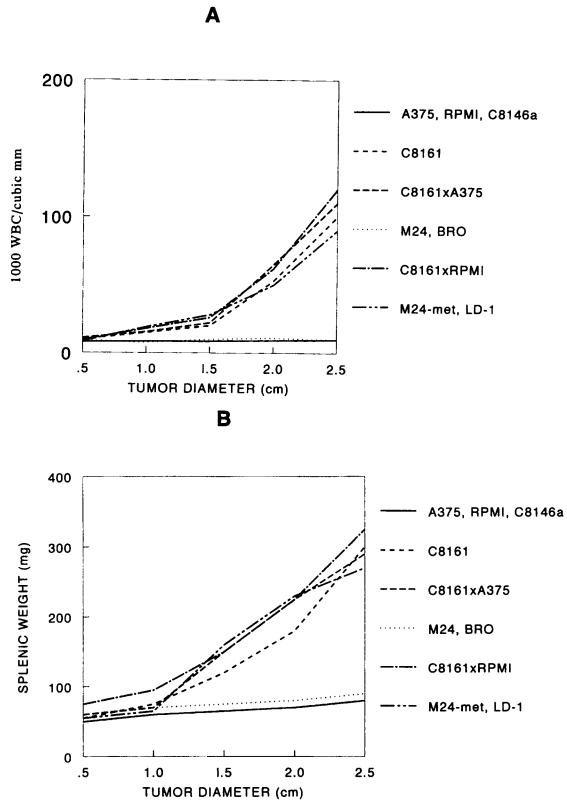

Figure 2. *Manifestations of Scid myelopoiesis.* A: *Effects on granulocytosis of different melanoma lines and C8161 fusions when grown as xenografts. Leukocyte counts were determined on peripheral blood with a Coulter counter adjusted to measure murine white blood cell parameters. A peripheral blood smear confirmed that >99% of the white blood cells in those xenografts showing increased white blood cells were mature granulocytes. Only C8161, LD-1, M24 met, and fusions involving C8161 as one parent showed induction of granulocytosis. The degree of granulocytosis strongly correlated with tumor size.* B: *Effects of the same melanoma lines and C8161 fusions on spleen size. Only C8161, LD-1, M24-met, and fusions involving C8161 as one parent showed marked splenomegaly. Splenic weight directly correlated with tumor size.*

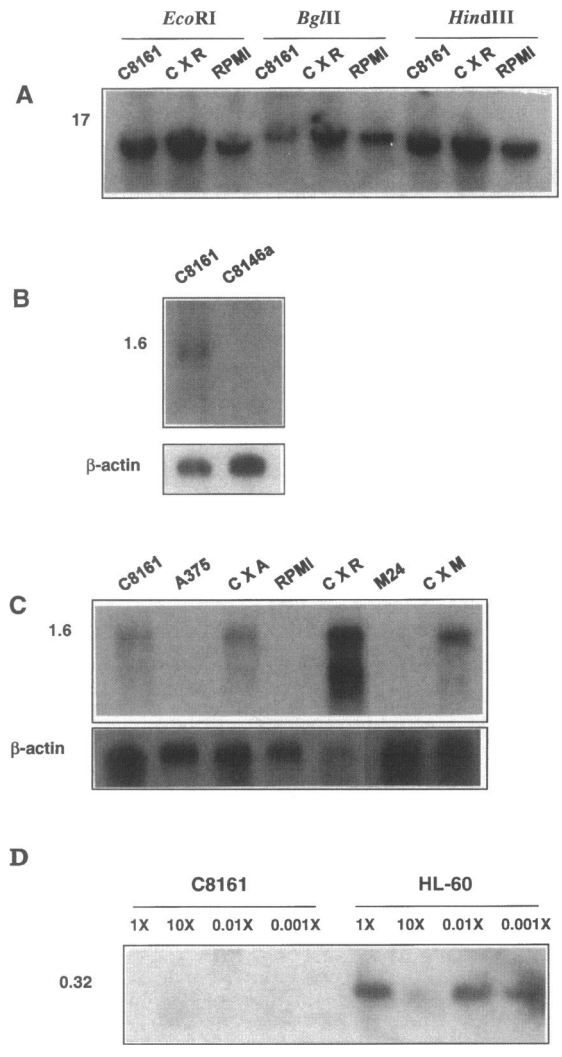

Figure 3. *G-CSF Southern/Northern blots and RT-PCR of G-CSF receptor.* A: *Southern blot of C8161, one typical non-G-CSF-expressing melanoma cell line, RPMI, and a hybrid clone, C8161 × RPMI (CXR). Twenty micrograms of genomic DNA was digested with 20 U of* EcoRI, BglII, *or* HindIII *for 19 hours, fractionated on a 0.8% agarose gel, blotted to a nylon membrane, and hybridized to a 0.7-kb* α-^{32}P-*labeled* PstI *fragment of plasmid containing human G-CSF cDNA. The characteristic bands ranging from 14 to 17 kb appeared. The banding pattern was identical in C8161, RPMI, and the hybrid clone. LD-1, M24-met, all of the C8161 hybrids, and all of the other non-G-CSF-expressing cell lines showed identical results. No gross amplifications or rearrangements of the human G-CSF gene were apparent.* B: *Northern blot of C8161 and C8146a demonstrate the characteristic 1.6-kb G-CSF transcript in only the G-CSF-expressing cell line C8161. LD-1 and M24 met gave similar results as C8161.* C: *M24, RPMI, and A375 gave similar results as C8146a. The C8161 hybrids, C8161 × A375 (CXA), C8161 × RPMI (CXR), and C8161 × M24 (CXM) revealed even more intense 1.6-kb bands than C8161 alone.* D: *Measurements of G-CSF receptor with RT-PCR. Amplified samples were run on a 2% agarose gel, transferred to a nylon membrane, and hybridized to a 2.9-kb G-CSF receptor probe. The blot demonstrated a 0.32-kb DNA signal only in HL-60 (positive control) when various concentrations of first-strand cDNA (2.4 μg (10X), 0.24 μg (1X), 0.0024 μg (0.01X), and 0.00024 μg (0.001X)) were used in amplification. No signals were detected in C8161. All of the C8161 hybrids as well as LD-1 and M24-met were also negative.*

whether the increased transcription might involve *trans*-dominant factors acting on the G-CSF promoter.

The somatic cell hybridizations that were carried out produced a number of clones that emerged from the double antibiotic selection (Table 1). At least five clones ultimately emerged from each fusion that by flow cytometric and chromosomal analyses were completely fused. These clones remained stable and retained 95 to 100% of their chromosomal complement through subsequent *in vitro* and animal passages. Self fusions, eg, *neo*C8161 × *hyg*C8161 and *neo*RPMI × *hyg*RPMI, showed no alteration in any phenotypic traits, including G-CSF expression and metastatic ability, compared with their respective parents. Hence, altered G-CSF expression or altered metastatic ability as an artifact of the polyethylene glycol fusion *per se* could be excluded. Somatic cell fusions of the C8161 line with the series of non-G-CSF-expressing lines not only produced hybrids that expressed G-CSF, but also, the levels of G-CSF produced by the hybrids were 3- to 10-fold greater than that of the C8161 line itself ($P < 0.01$; Figure 4, A and B). These observations held for all five or more of the completely fused clones that were obtained from each fusion. The regulation of this enhanced G-CSF expression in the hybrids was again transcriptional (Figure 3C).

The observations in the somatic cell hybridization experiments suggested that the mechanism of increased G-CSF transcription and expression in the C8161 hybrids possibly involved *trans*-dominant factors acting on the G-CSF promoter. For this reason we conducted G-CSF promoter/CAT reporter transfections of the various G-CSF-expressing and G-CSF-non-expressing cell lines and hybrids. CAT expression in the G-CSF-expressing cell lines ranged from 1.0×10^{-3} to 1.65×10^{-3} ng/μg cellular protein. These values reflected a three- to fivefold increased CAT over baseline (promoterless pBLCAT3 transfection). The non-G-CSF-expressing cell lines showed either baseline or below baseline values (Figure 4C).

The somatic cell fusions of C8161 with the non-G-CSF-expressing melanoma lines involved fusions between an undifferentiated melanoma line, C8161, a line that lacked markers of melanocytic differentiation,[10] and a series of melanoma lines of higher differentiation status expressing one or more of the common melanocytic markers, S100, HMB-45, and NKI/C3. The hybrids demonstrated a C8161-dominated phenotype where the expression of all of the melanocytic markers was completely extinguished, and instead, the expression of epithelial keratins 8

Table 1. *Correlation of G-CSF Expression and Spontaneous Metastasis*

Cell lines	Chromosomes		G-CSF	G-CSF receptor	Tumorigenicity	Metastasis
	Mode	Range				
C8161	82	69–95	+	–	10	10
M24met	57	40–78	+	–	10	8
LD-1	N.T.		++	–	10	7
C8146a	75	65–85	–	–	9	0
A375	58	48–68	–	–	9	0
RPMI	64	56–70	–	–	10	1
SKMEL	100	85–105	–	–	5	0
M24	60	45–69	–	–	9	1
M15	87	65–100	–	–	4	0
BRO	110	85–140	–	–	10	3
C8161 × C8146a	155	125–175	++	–	9	9
C8161 × A375	136	102–152	+++	–	10	9
C8161 × RPMI	140	135–155	++++	–	9	9
C8161 × SMEL	175	145–205	++	–	8	8
C8161 × M24	140	125–160	++	–	10	10
C8161 × M15	167	149–178	++	–	8	8
C8161 × BRO	192	160–215	+++	–	10	10

Chromosome number (mode and range determined by counting 20 metaphase spreads), G-CSF expression, and G-CSF receptor (R) expression in the various melanoma lines and hybrids were determined. For the hybrids, a representative clone is depicted from each fusion; G-CSF expression is depicted in relative amounts: +, ++, +++, and ++++; –, not detected; NT, not tested. Tumorigenicity and metastasis are expressed as numbers of mice of 10 showing primary tumors and metastasis, respectively.

and 18, markers of C8161's undifferentiated phenotype, was maintained (Table 2). The hybrids were all highly metastatic, producing several hundred pulmonary and other visceral metastases in 80 to 100% of the mice when the primary tumor reached 1.5 to 2.0 cm in size. There was a strong correlation observed in the individual melanoma and hybrid cell lines between G-CSF expression and spontaneous metastasis (Table 1). Although the non-G-CSF-expressing cell lines, including M24, showed either no or low levels of metastasis, the C8161, C8161 hybrids, LD-1, and M24-met, a subclone of M24,[20] all were both highly G-CSF expressive and highly metastatic ($P < 0.01$). There were no significant alterations in susceptibility to either natural killer cell or macrophage cytolysis (stimulated or unstimulated) among the C8161, LD-1, M24-met, C8161 hybrid, and non-G-CSF-expressing cell lines. All of the cell lines studied exhibited relatively low susceptibility to natural killer cell and macrophage cytolysis (data not shown).

To explain the strong correlative relationship between G-CSF expression and spontaneous metastasis, the presence of either an autocrine or paracrine loop was investigated. The presence of an autocrine loop involving G-CSF and its receptor, which could account for the enhanced growth and hence metastasis-forming ability of the G-CSF-expressing cell

Table 2. *C8161 Dominance of Its Undifferentiated Phenotype*

Cell lines	C	S-100	HMB-45	NKI/C3	Mel
Parents					
C8161	+++	–	–	–	–
SKMEL	–	+++	+++	+++	+++
M15	–	–	–	+++	–
A375	–	++	–	+++	–
BRO	–	–	–	+++	–
RPMI	–	–	–	+++	+
M24	–	–	–	+++	–
C8146a	–	+++	+++	+++	–
Hybrids					
C8161 × SKMEL	+++	–	–	–	–
C8161 × M15	+++	–	–	–	–
C8161 × A375	+++	–	–	–	–
C8161 × BRO	+++	–	–	–	–
C8161 × RPMI	+++	–	–	–	–
C8161 × M24	+++	–	–	–	–
C8161 × C8146a	+++	–	–	–	–

As in Table 1, a representative clone is depicted from each fusion; C, cytokeratin; Mel, melanin; immunoreactivity is depicted in relative amounts: +, ++, and +++; –, not detected.

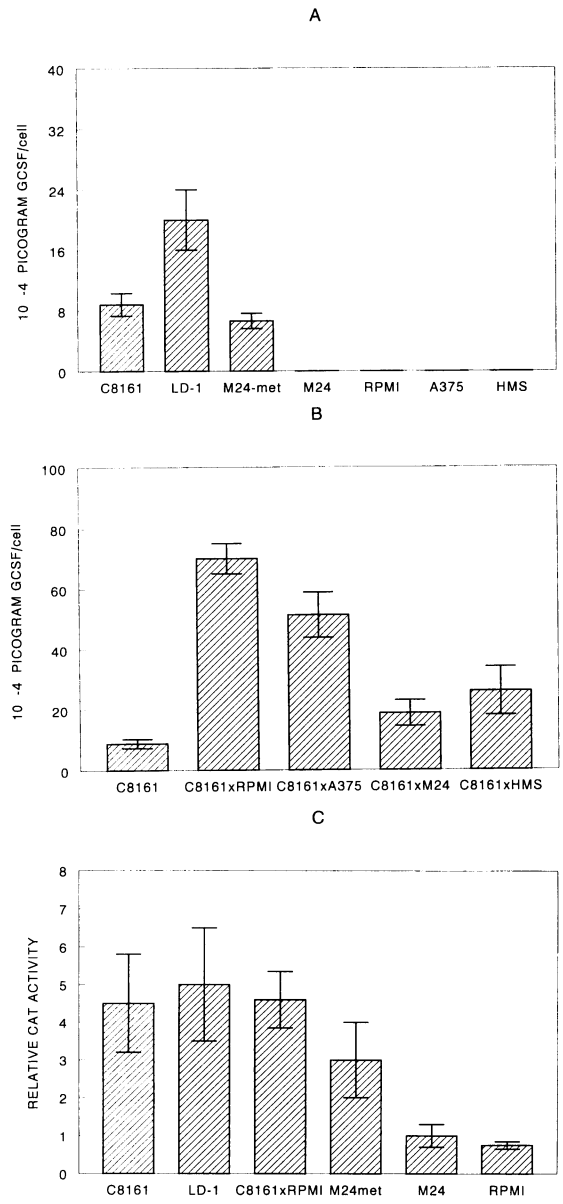

Figure 4. *G-CSF and CAT expression detected by ELISA in the different cell lines and hybrids.* A: *G-CSF was detected in high amounts in the metastatic C8161, LD-1, and M24-met lines but not in the nonmetastatic lines M24, RPMI, A375, or HMS-1.* B: *Fusions of C8161 with the non-G-CSF-expressing cell lines exhibited 3- to 10-fold greater G-CSF expression than that exhibited by C8161 itself.* C: *Increased CAT expression was observed in the G-CSF-expressing cell lines; the level of CAT expression in the pBLCAT3 transfectants was assigned a value of 1 and the levels in the pGCAT transfectants are shown relative to this value. The relative CAT levels depicted are the mean ± SD of three separate transfection experiments.*

lines and hybrids, was sought by RT-PCR studies of the G-CSF receptor. No G-CSF receptor transcripts could be demonstrated in the C8161 line, LD-1, M24-met, or any of the C8161 hybrids (Figure 3D). Hence, the presence of an autocrine loop seemed unlikely. Next, the presence of a paracrine loop involving G-CSF stimulation of some murine host factor(s) that in turn might stimulate C8161, LD-1, or M24-met growth and metastasis was sought. In the G-CSF Scid injection experiments (Figure 5, A–D), no differences in xenograft latency, growth, or metastasis were observed in the G-CSF-treated *versus* G-CSF-non-treated C8161, LD-1, M24 met, and C8161 hybrids ($P > 0.25$). All of the nonmetastatic lines remained nonmetastatic and their latency and growth also remained unchanged with G-CSF treatment.

In the somatic cell hybrid experiments, as our results ran counter to previous studies that the more malignant and undifferentiated properties are usually suppressed in fusions with less malignant and more differentiated cells, the claim that our hybrids represented true and complete fusions and not improperly fused subsets of C8161 was intensely verified by chromosomal marker studies, polymorphic genetic loci, DNA ploidy studies, and control fusions. Under the double-selective conditions applied to each parent alone, a log kill $\geq 10^6$ was achieved, eliminating all traces of each parent. No clones were therefore observed on control plates from self fusions, eg, *neo*C8161 × *neo*C8161 or *hyg*A375 × *hyg*A375. For the varying fusions involving different parents, 30 to 100 clones emerged. Of these, approximately 50% either ceased dividing or were not recovered after trypsinization. Another 30% were incompletely fused. At least five clones eventually emerged from each fusion that were completely fused on the basis of DNA ploidy and karyotype analysis. Hence, each of the cell fusions yielded stable and completely fused somatic cell hybrids at a frequency of at least 5×10^{-6}. The hybrids were morphologically different from both parents in every case. By karyotype analysis, the presence of marker chromosomes from each parent was observed. For example, in the C8161 × HMS-1 fusions, the presence of a rearranged marker chromosome (t(6;9)(p11.2;p13)) of HMS-1[21] was verified to be present in the fusions. Therefore, on the basis of all of these findings, the apparent dominance of the C8161 phenotype in the fusions was not an artifact of an improperly fused subset of C8161 but rather a reflection of true genetic dominance presumably mediated by a *trans*-acting transcriptional pathway(s).

The *trans*-acting pathway(s) responsible for the metastatic and the enhanced G-CSF-expressing phenotype of the hybrids did not, however, involve genes that had been implicated previously in tumor progression and metastasis as either positive or negative regulators (Figure 6, A–C). Levels of *nm23* were either elevated or showed no alterations in the G-CSF-expressing metastatic hybrids compared with the non-G-CSF-expressing nonmetastatic parents

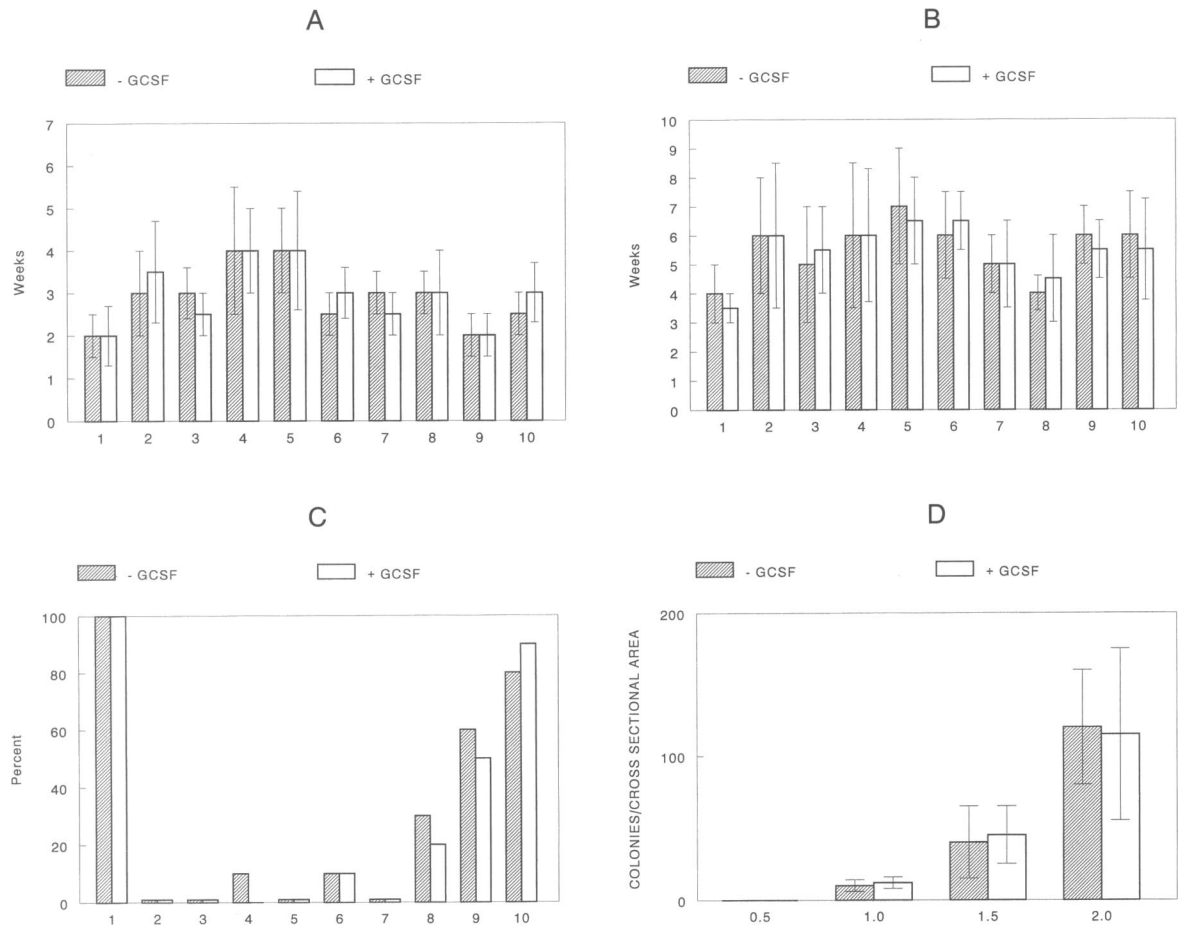

Figure 5. *Parameters of tumor progression with (+) and without (−) G-CSF.* **A:** *Xenograft latency period. The latency period was defined as the time period between injection of the cells and the first emergence of a visible tumor.* **B:** *Xenograft growth. The growth of the xenograft is expressed as the time after the first emergence of a visible tumor to achieve a 2.0-cm-diameter tumor nodule. In* **A** *and* **B**, *data depict mean ± standard deviation in groups of 10 mice.* **C:** *Spontaneous metastasis at 6 weeks after injection expressed as percentage of mice with pulmonary metastasis:* **1**, *C8161;* **2**, *C8146a;* **3**, *A375;* **4**, *RPMI;* **5**, *SKMEL;* **6**, *M24;* **7**, *M15;* **8**, *BRO;* **9**, *LD-1;* **10**, *M24-met.* **D:** *C8161 pulmonary metastasis (colonies/cross-sectional area) versus primary size (maximal diameter in centimeters). The number of metastatic colonies in mid-longitudinal cross sections of one lung was determined by digital image analysis of five different cross sections. Modal numbers (and ranges) are depicted. G-CSF exerted no effects on decreasing xenograft latency or increasing xenograft growth. G-CSF likewise exerted no effects on metastasis.*

(Figure 6A); levels of DNA methyltransferase were either decreased or showed no alterations in the G-CSF-expressing metastatic hybrids compared with the non-G-CSF-expressing nonmetastatic parents (data not shown), and levels of epidermal growth factor receptor (EGFR) by immunocytochemical analysis were increased in both the G-CSF-expressing metastatic hybrids as well as the C8161 line and the non-G-CSF-expressing nonmetastatic parents and thus showed no differential expression (Figure 6C). *p53* alterations were not detected by immunocytochemical analysis in either the C8161, the C8161 hybrids, or any of the non-G-CSF-expressing nonmetastatic lines studied (Figure 6B). A normal p53 exon pattern by single-strand conformation polymorphism confirmed the p53 immunocytochemical data (data not shown).

Discussion

Human G-CSF is one of four CSFs that belong to a family of glycoproteins that control the survival, proliferation, differentiation, and functional activation of hematopoietic progenitor cells.[38–41] G-CSF may have, however, other nonhematopoietic actions.[42] Ectopic G-CSF expression by several different human and murine tumor cell lines of diverse lineages has been noted previously. These tumor cell lines have included the human squamous cell carcinoma line CHU-2, the human bladder carcinoma line 563, the human adenocarcinoma line SK-HEP-1, a murine fibrosarcoma line, a human glioblastoma line, U87MG, and human melanoma lines.[7,8,43–47] Furthermore, in select clinical case reports, the presence of marked granulocytosis in diverse tumor

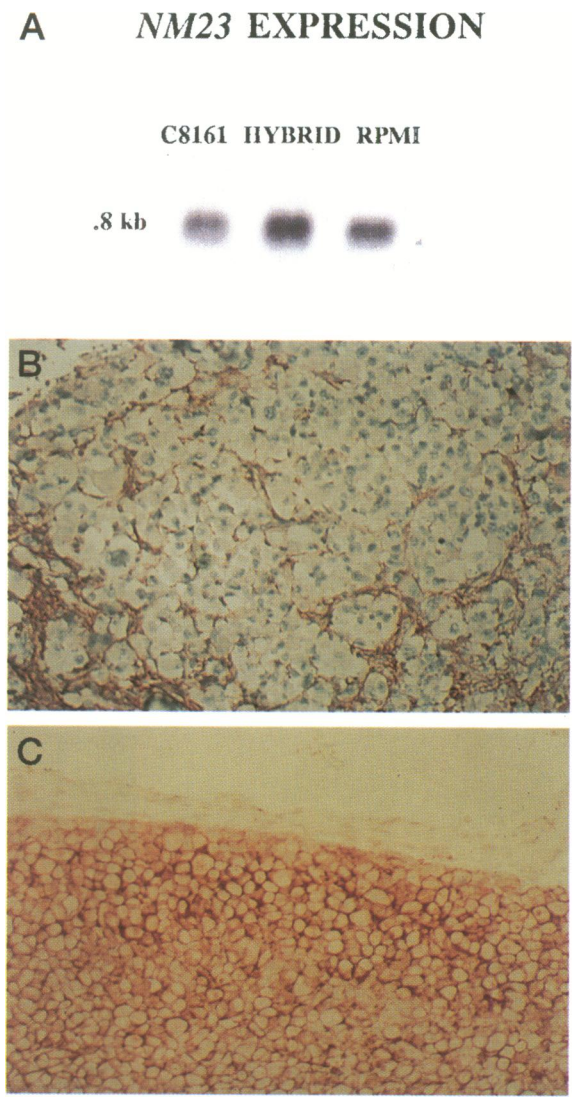

Figure 6. *The C8161 × RPMI hybrid is depicted with respect to nm23, p53, and EGFR.* A: *Northern blot shows a two- to fourfold increase in nm23 in this hybrid over that expressed by the nonmetastatic RPMI.* B: *Negative p53 immunoreactivity was observed in this hybrid. Anti-p53; immunoperoxidase; magnification, ×400.* C: *Increased membrane EGFR immunoreactivity was present in this hybrid as well as in the nonmetastatic RPMI. Anti-EGFR; immunoperoxidase; magnification, ×250.*

types associated with ectopic G-CSF expression *in vivo* has been observed. Our observations note ectopic G-CSF production in three different human melanoma lines, C8161, LD-1, and M24-met. Although ectopic G-CSF production by melanoma cells has been noted previously,[7,8] the question of the mechanism behind the ectopic synthesis has not been addressed. Although gross gene rearrangements and alternate splicing have been identified in one cell line, CHU-2, as a possible mechanism of enhanced G-CSF expression,[43] our observations in C8161, LD-1, M24-met, and the C8161 hybrids would suggest that gross gene rearrangements or amplifications were not the mechanisms of increased G-CSF expression. Our studies with the somatic cell hybrids of C8161 show a 3- to 10-fold increase in G-CSF expression over that of C8161 and our transfection studies with pGCAT show a 3- to 5-fold increase in reporter activity in all of the G-CSF-expressing lines and hybrids. These studies provide support for a dominant *trans*-acting mechanism. Our studies do not entirely exclude co-existent *cis* alterations in the G-CSF promoter of C8161, M24-met, and LD-1 leading to increased expression from normal transcription factors that would otherwise be inactive with a normal promoter, but we consider this explanation less likely for the following reasons. Our reporter constructs utilized a 330-bp promoter fragment (−311 to +18) that had been cloned by PCR from genomic DNA prepared from Jurkat T cells, a cell line that contains no known *cis* alterations of the G-CSF promoter. These reporter constructs demonstrated a 3- to 5-fold increase in CAT activity over baseline in only those melanoma lines, LD-1, M24-met, C8161, and its fusions, that expressed ectopic G-CSF. The non-G-CSF-expressing melanoma lines' parents did not express CAT above baseline. We realize that these studies do not totally exclude co-existent *cis* alteration within the human G-CSF promoter of the melanoma lines being studied, but considering that three different lines show increased expression of G-CSF as well as increased CAT and that all of the C8161 fusions show increased G-CSF as well as increased CAT, we feel that it is unlikely that co-existent promoter *cis* alterations are playing a significant role in G-CSF expression in all of these lines. Rather, the data convincingly support the presence of *trans*-acting factors that are mediating G-CSF expression. Even if co-existent *cis* alterations were present together with *trans*-acting factors, this would not detract from our central hypothesis that G-CSF expression is marking a *trans*-dominant transcriptional pathway of tumor progression. In previous studies with pGCAT,[28] the 330-bp promoter fragment was observed to induce CAT reporter gene expression in appropriate human cell lines in response to the inducers tumor necrosis factor-α or interleukin-1β. This promoter region (−311 to +18) has been shown to contain at least three regulatory essential promoter elements that mediate expression of G-CSF in response to tumor necrosis factor-α and interleukin-1β.[28] In addition, an equivalent region of the murine G-CSF promoter transfected into human carcinoma cells has demonstrated that human nuclear factors exist that bind to each region and probably activate transcription.[44] For these reasons and

the availability of pGCAT, we chose to carry out our transfection studies with this region of the G-CSF promoter. The CAT reporter activity was similar in all of the G-CSF-expressing lines and hybrids even though the levels of G-CSF expression varied up to 10-fold more in the C8161 hybrids (Figure 4, B and C). This may indicate that other *trans*-acting factors are involved that bind to other regions of the G-CSF promoter not contained within the 330-bp promoter region of pGCAT.

Our somatic cell hybrids demonstrated not only dominance of the G-CSF-expressive phenotype but also dominance of both an undifferentiated as well as metastatic phenotype (Tables 1 and 2). Most studies with somatic cell hybrids have observed that the more differentiated phenotype of the parents predominates in the fusions. For example, in hybrids produced by somatic cell fusions of MCF-7 with a normal immortalized human mammary epithelial line, features of the more normal parent, which included loss of tumorigenicity, increased extracellular matrix gene expression, and regression of the MCF-7 phenotype, predominated.[48] In another somatic cell fusion between a human lung carcinoma line and nontumorigenic bronchial epithelial cells, there again was suppression of tumorigenicity.[49] Although it might have been anticipated then that our hybrids similarly would show features of the more normal parent, which would include suppression of G-CSF expression and absence of metastasis, our hybrids demonstrated exactly the opposite, showing enhanced expression of G-CSF and enhanced metastasis formation. Our hybrids then demonstrate a dominant *trans*-acting pathway.

The mechanism of C8161's dominance did not involve genes previously implicated in tumor progression and metastasis, eg, the negative regulators *nm23* and *p53* and the positive regulators DNA methyltransferase and EGFR.[19,50–52] Therefore, the pathway(s) operating in our hybrids linked to ectopic G-CSF expression and metastasis may involve novel genes and gene products.

What then is the significance of ectopic G-CSF expression in the C8161 and its hybrids and what is its relationship, if any, to tumor progression and metastasis? Is ectopic G-CSF expression a mere epiphenomenon or is there a relationship to tumor progression? Does the observation of ectopic G-CSF production by C8161 have relevance to the general issue of ectopic hormone or growth factor production by human tumors? The presence of an autocrine loop involving G-CSF and its receptor was sought because it could account for the relationship between G-CSF expression and enhanced growth and metastasis-forming ability exhibited by all of the G-CSF-expressing cell lines and hybrids. There was also precedence for hematopoietic growth factor receptors on various nonhematopoietic tumors such as melanoma[53,54] and precedence for such autocrine loops in the development and progression of melanoma.[55,56] However, with the sensitive RT-PCR technique, we found no evidence of the typical G-CSF receptor (Figure 3D). Although these findings do not completely exclude the presence of another yet uncharacterized receptor, it is unlikely that G-CSF is functioning as a ligand for the autocrine stimulation of C8161 and its hybrids, which could account for their enhanced growth and metastasis-forming properties.

What about a paracrine effect? Recent studies have observed a positive paracrine effect of granulocytes on the growth of select tumor cell variants and a positive effect of granulocytes on heterotypic clumping of tumor cells in the circulation, which may promote hematogenous metastasis.[57] However, these studies examined the effects of granulocytes on tumor cell parameters by depleting granulocytes from the mice with an antigranulocyte antibody; the effects of a pronounced granulocytosis on tumor cell growth and metastasis were not examined. In our study, the experiments examining the effects of injected rG-CSF and the resulting granulocytosis on tumorigenicity and metastasis of the C8161 and the other cell lines demonstrated that a paracrine stimulatory effect between G-CSF and parameters of tumor progression and metastasis of these lines did not exist. The lack of a demonstrable paracrine effect of G-CSF on metastasis of human melanomas in Scid mice must be viewed cautiously, however, because our system involves an immunodeficient mouse. Thus, certain potential paracrine effects of G-CSF on human melanoma growth and metastasis in the natural immunocompetent host might not be appreciated in our system and hence should not be completely dismissed.

Because the increased G-CSF expression was due to a *trans*-dominant pathway as illustrated in the somatic cell hybridization studies and pGCAT reporter studies of a number of different melanoma lines and because human melanoma has been observed occasionally to produce granulocytosis *via* G-CSF production *in vivo,* the explanation that what we are observing in the present study is either an idiosyncratic or stochastic event of genetically unstable cell lines seems unlikely. Nonrandom events, which characterize tumor progression in general, usually provide selective growth advantages to the tumor cells. Because such growth advantage from

ectopic G-CSF was not apparent from either an autocrine or paracrine perspective, the *trans*-dominant pathway responsible for G-CSF expression may be activating other effector molecules, as yet unidentified, that are causally linked to the progression of these melanoma lines and perhaps some clinical melanomas. The hypothesis that ectopic hormone expression is a marker of a higher-level transcriptional pathway has direct relevance to all human cancers where ectopic hormone or growth factor production occurs *in vivo* for which there is no obvious benefit to the tumor. If these conclusions are correct, using either a reverse strategy beginning with the G-CSF promoter to identify *trans*-activating DNA-binding proteins that are present in C8161, LD-1, M24-met, and the C8161 hybrids and working backwards or using a forward strategy with a eukaryotic expression library made from C8161, LD-1, or M24-met transfected into the non-G-CSF-expressing recipient cell lines followed by G-CSF screening to identify genes that positively regulate G-CSF expression might prove effective in identifying the key molecules of this *trans*-dominant pathway that are involved in tumor progression and metastasis.

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