

Interleukin-6 Undergoes Transition From Paracrine Growth Inhibitor to Autocrine Stimulator during Human Melanoma Progression

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Abstract. The ability to penetrate the dermal basement membrane and subsequently proliferate in the underlying mesenchyme is one of the key steps in malignant progression of human melanomas. We previously undertook studies aimed at assessing how normal dermal fibroblasts (one of the main cellular components of mesenchyme) may affect the growth of human melanoma cells and facilitate the overgrowth of malignant subpopulations (Cornil, I., D. Theodorescu, S. Man, M. Herlyn, J. Jambrosic, and R. S. Kerbel. 1991. *Proc. Natl. Acad. Sci. USA.* 88:6028–6032). We found that melanoma cell lines from early-stage (metastatically incompetent) lesions were growth inhibited whereas those from advanced-stage (metastatically competent) lesions were stimulated under the same conditions by co-culture with fibroblasts; conditioned medium from such cells gave the same result. Subsequent studies using biochemical purification and neutralizing antibodies revealed the inhibitory activity to be identical to interleukin-6 (IL-6). We now report that addition of purified recombinant human IL-6 resulted in a growth inhibition in vitro by G1/G0 arrest of early, but not advanced stage melanoma cells. Despite this alteration in response there was no significant difference in melanoma cell lines of varying malignancy in respect to their expression of genes encoding the IL-6 receptor, or gp130, the IL-6 signal transducer. Scatchard analysis also revealed similar [¹²⁵I]IL-6 binding activities in both IL-6 sensitive and resistant groups. However, studies of IL-6 production indicated that five out of eight IL-6 melanoma cell lines known to be resistant to exogenous IL-6-mediated growth inhibition constitutively expressed mRNA for IL-6; they also secreted bioactive IL-6 into culture medium. To assess the possible role of this endogenous IL-6 in melanoma cell growth, antisense oligonucleotides to the IL-6 gene were added to cultures of melanoma cells. This resulted in a significant growth inhibition only in cell lines that produced endogenous IL-6. In contrast, neutralizing antibodies to IL-6 were ineffective in causing such growth inhibition. This indicates that endogenous IL-6 may behave as a growth stimulator by an intracellular (“private”) autocrine mechanism. Thus, a single cytokine, IL-6, can switch from behaving as a paracrine growth inhibitor to an autocrine growth stimulator within the same cell lineage during malignant tumor progression. Such a switch may contribute to the growth advantage of metastatically competent melanoma cells at the primary or distant organ sites and thereby facilitate progression of disease.

One of the mechanisms by which cancer cells are thought to acquire a growth advantage is through the “autocrine” production of mitogenic growth factors (2, 48). This refers to the process whereby cancer cells, or their dysplastic cellular precursors, begin to express a growth factor (or receptor for the growth factor) which their normal cellular counterpart does not normally express. The receptors may be cell surface associated or expressed intracellularly, thereby creating “public” or “private” autocrine loops, respectively (4). In the case of solid tumors, whether carcinomas, sarcomas, or tumors of neuroectodermal origin (e.g., brain tumors, malignant melanoma), the nature of many of these autocrine growth factors involved in their growth is well known (2). They include, for example, members of the FGF family such as basic FGF (bFGF), PDGF, transforming growth factor- α (TGF- α),¹ EGF, and the insulin-like growth factors, IGF-I and IGF-II. Conspicuously absent from this list are members of the hemopoietic family of cytokines and growth factors (13, 35). These include the interleukins (ILs), colony stimulating factors (CSFs), erythropoietin and stem cell factor (SCF). Not surprisingly, these factors have been studied predominantly as paracrine or autocrine regulators of the growth of leukemias and lymphomas (2, 13). However, there is a small (but growing) litera-

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1. *Abbreviations used in this paper:* CM, conditioned media; IL, interleukin; rhIL-6, recombinant human IL-6; TGF, transforming growth factor.

ture suggesting that members of the hemopoietic growth factor family may also help regulate the growth of solid tumors directly, especially in a paracrine manner (5, 9, 10, 39, 45). Prominent among these factors may be interleukin-6 (IL-6).

IL-6 is produced by a variety of cells including fibroblasts, endothelial cells, keratinocytes (all of which make this a potentially interesting cytokine for skin cancers, including melanoma), T and B lymphocytes and monocytes/macrophages (18, 27, 57). It is thought to be a major molecular mediator of inflammatory conditions, acute phase protein synthesis in the liver, and various immunological reactions (27). With respect to hematologic cancers it has been shown, for example, to behave as an autocrine growth factor for some lymphomas and human multiple myelomas in culture (24, 29, 40, 65).

We recently uncovered evidence to show IL-6 can affect the growth properties of human melanomas differentially as a function of tumor progression (30). Thus, the growth of cell lines established from early-stage benign (curable) primary melanomas was inhibited by co-culture with normal dermal fibroblast or conditioned media from such cells (8). In contrast, cell lines established from more advanced (malignant) primary melanomas, or metastases, were stimulated under the same conditions (8). The activity responsible for the growth inhibition of early-stage melanoma cells was subsequently identified as being IL-6 (30); the nature of the stimulator has not yet been determined. Consistent with these conclusions was the finding that addition of nanogram amounts of recombinant human IL-6 to early-stage melanoma cells was profoundly inhibitory to their growth whereas cells from more advanced cell lines were completely resistant to this inhibition (30).

The purpose of the present studies was to investigate the possible reasons for the transition in responsiveness to IL-6 during melanoma progression. Such a transition could provide malignant (i.e., metastatically competent) melanoma cells with a growth advantage, helping them to acquire the ability to proliferate in the foreign environment of the dermal mesenchyme (6), and thus achieve 'clonal dominance' (25). We examined the IL-6 receptor status, and IL-6 production itself, among different melanoma cell lines obtained from different stages of disease progression. We found evidence that IL-6 is produced by >50% of the advanced stage cell lines, and surprisingly, that it can function as an intracellular autocrine growth factor in such cells. Thus a single growth factor can change from functioning as a (paracrine) inhibitor to an autocrine stimulator within the same cell lineage during the multi-step process of malignant tumor progression.

Materials and Methods

Cell Culture and Growth Assay

The human melanoma cell lines used in these studies are the same as described previously (8, 14, 15, 30). The cell lines in RPMI 1640 medium containing 5% FBS (8, 30). With the exception of SKMEL28 and MeWo, the cell lines were established from different lesional stages of disease progression in the laboratory of Dr. M. Herlyn (Wistar Institute, Philadelphia, PA) (14, 15). Some were derived from early-stage nonmalignant primary tumors, i.e., from radial growth phase (RGP) or early ("thin") vertical growth phase (VGP) primary melanomas. Patients were cured by surgical removal of these lesions. Other cell lines were authentic "malignant" (i.e.,

metastatically competent) melanomas. They were derived from either advanced local VGP primaries where there was evidence that the patient had distant metastases at the time the primary lesion was removed, or from distant metastases (14, 15). In experiments designed to assess cell proliferation by [³H]thymidine incorporation, 5×10^3 cells were plated into 96-well plates with or without recombinant human IL-6 (rhIL-6) (Upstate Biotechnical Inc., Lake Placid, NY) in triplicate, using ExCell 300 medium (J. R. Scientific, Woodland, CA) and 1% FBS. Cells were cultured at 37°C for 2 d. 50 μ l of 2.0 μ Ci [³H]thymidine (25 Ci/mmol; Amersham Corp., Arlington Heights, IL) was added to culture wells and cells incubated for another 4–6 h before being harvested in a Titertek Cell Harvester 530 with Printed Filtermat A (Pharmacia Fine Chemicals, Piscataway, NJ). Radioactivity in the harvested filters was counted in 1205 Betaplate™ scintillation counter (Wallac, Gaithersburg, MD).

Flow Cytometric DNA Analysis

Human melanoma cells (1×10^6 cells) were plated overnight in 100-mm dishes in RPMI 1640 medium containing 5% FBS. The culture medium was then changed to RPMI 1640 medium containing 2% FBS without or with rhIL-6 at 20 ng/ml concentration for 24 h. Cells were harvested by trypsinization and washed three times with PBS. The cell pellets were then frozen and nuclei prepared as described previously (58, 59). DNA content was measured in EPICS Elite flow cytometer (Coulter Electronics, Inc., Hialeah, FL) using propidium iodide staining (58, 59) and the data were analyzed with the software program provided by the supplier.

Scatchard Analysis of IL-6 Receptors

Determination of the affinities and sites of IL-6 binding to human melanoma cell lines was conducted essentially as described before by Taga et al. (51). Briefly, purified carrier-free rhIL-6 (20.3 kD, purchased from R & D Systems, Minneapolis, MN) was radio-iodinated with Bolton-Hunter reagent (Amersham Corp.). [¹²⁵I]IL-6 was separated from free iodine by Sephadex G-50 column chromatography. The specific activity of [¹²⁵I]IL-6 was 4.4×10^{13} cpm/g as determined in a human myelomonocytic leukemia cell line, called CESS (American Type Culture Collection, Rockville, MD) by self-displacement analysis. Incubation of [¹²⁵I]IL-6 with human melanoma cells ($0.8\text{--}1.3 \times 10^6$) was carried out in ice (0°C) for 150 min in a final volume of 70 μ l in RPMI 1640 medium containing 20 mM Hepes, pH 7.4, and 1 mg/ml BSA. Specific binding of [¹²⁵I]IL-6 to human melanoma cells was calculated after subtracting from the total binding counts of samples with 250 ng of unlabeled rhIL-6 (Upstate Biotechnology Inc.) i.e., the nonspecific binding. Experiments were done in duplicate. Data are expressed by mean \pm standard error of three separate experiments.

Northern Blotting Analysis of mRNA

Poly(A)⁺ RNA was prepared by use of oligo (dT) cellulose (3) from human melanoma cell lines that were cultured in RPMI 1640 medium containing 5% FBS. For Northern blot analyses, 3–6 μ g of poly(A)⁺ RNA was size-fractionated in 1% formaldehyde agarose gels. Hybridizations were conducted at 65°C overnight using ³²P-labeled cDNA probes. cDNA probes for human IL-6 (TaqI/BanII fragment), IL-6 receptor (FspI/EspI fragment), gpl30 (AccII/BamHI fragment) and human liver glyceraldehyde-3-phosphate dehydrogenase (GAPDH, PstI/XbaI fragment, American Type Culture Collection) were radiolabeled with ³²P-dCTP and Klenow fragment of DNA polymerase I and random hexamer primers (16, 17, 55, 62).

B9 Cell Bioassay for IL-6 Proliferative Activity

The IL-6-dependent murine B-cell hybridoma B9 cell line was used and cultured as described previously (1, 42). Conditioned media (CM) of human melanoma cell lines were collected and assayed as follows: melanoma cells were subcultured in 6-well plates until confluent in RPMI medium containing 5% FBS. A total of 2.5 ml of the same medium as well as fresh medium was then added into the culture overnight and CM was collected (called 'CM1') which contained 5% FBS. Subsequently, cells were washed once with PBS and cultured in 2.5 ml of ExCell 300 medium overnight. The serum-free medium (called CM2) was then collected. B9 cells were plated and cultured in 96-well plates at 10^4 cells per well, with or without CM obtained from melanoma cell cultures or rhIL-6 and/or anti-hIL-6 neutralizing antibody, at 37°C for 2 d. Cells were pulse-labeled with [³H]thymidine for 4–6 h before being harvested in glass fiber filters, as described above. Stimulating units of melanoma cell CM were calculated by the concentration of CM that doubled the [³H]thymidine uptake in B9 cells.

IL-6 Antisense Oligonucleotide-Mediated Inhibition of Cell Growth

A 15-base antisense oligodeoxynucleotide (TCCTGGGGGTACTGG) that is specific for a sequence of the second exon of IL-6 gene was used, as described by others previously (29, 34, 64). The antisense and a control 15-mer sense oligonucleotide were synthesized by Pharmacia Biotechnology Service Centre (Toronto, Ont.) and phosphorothioate preparations were made in the Department of Medical Genetics, University of Toronto. To assess the effect of antisense oligonucleotides on the growth of melanoma cell lines, cells were cultured in 24-well plates (10^4 cells per well) with or without 10 μ M of the oligonucleotides in RPMI 1640 medium containing 5% FBS for 5 d. [3 H]thymidine incorporation was conducted as described above with addition of oligonucleotide preparations. Cell numbers were counted by trypan blue exclusion in a hemocytometer. Cell number in wells without oligonucleotide was calculated as 100%.

Results

IL-6 Arrests Early-Stage Melanoma Cell Growth at the G1/G0 Boundary of the Cell Cycle

Previously, we had found that recombinant human IL-6 inhibited the proliferation of three out of four melanoma cell lines that were derived from early-stage (metastatically incompetent) primary lesions (30). These three cell lines were WM35, WM902B, and WM1341B; they are grouped and referred to in this paper as "IL-6 sensitive." The exception is the WM793 cell line, which is not growth inhibited by IL-6 (but which is, by exposure to IL-1 and TNF α). Among the seven human melanoma cell lines derived from either advanced-stage primary tumors, or metastases, none was growth inhibited significantly by the addition of IL-6 into the culture medium (30). These are WM1361A, WM983A, WM1205, WM9, WM451, MeWo, and SKMEL28, which (including WM793) are referred to as the "IL-6-resistant" group.

Flow cytometric DNA analysis was conducted in cell lines from both IL-6 sensitive and resistant groups of melanoma cells. As shown in Fig. 1 and Table I, addition of recombinant human IL-6 into culture medium resulted in a time-dependent growth inhibition of WM35 cell growth as assessed by [3 H]thymidine incorporation. This corresponded to a decrease in cell numbers in S phase, and to an increase in cell numbers in the G1 phase. Similar changes were also found in other cell lines that are sensitive to IL-6 induced growth inhibition (Table I). The results suggest that IL-6 induced growth inhibition of early-stage melanoma is mediated by arrest in the G1/G0 boundary of the cell cycle. As expected, changes of cell ratio in G1 and S phase upon addition of IL-6 were not observed in IL-6 resistant, advanced-stage, malignant melanoma cell lines.

Evaluation of IL-6 Receptor Status in Melanoma Cell Lines

Northern blotting analysis revealed similar levels of IL-6 receptor gene expression in both IL-6 sensitive and IL-6 resistant cell lines (Fig. 2). However, it is known that IL-6 receptor itself does not possess signal transduction function and that IL-6 action is mediated through "gp130" after IL-6 binds to its receptor (16, 38). We therefore examined the levels of mRNA for gp130 but did not find any marked difference in gp130 gene expression among these human melanoma cell lines (Fig. 2). This suggests (but does not prove) that defective gp130 or altered expression of gp130, is not involved in

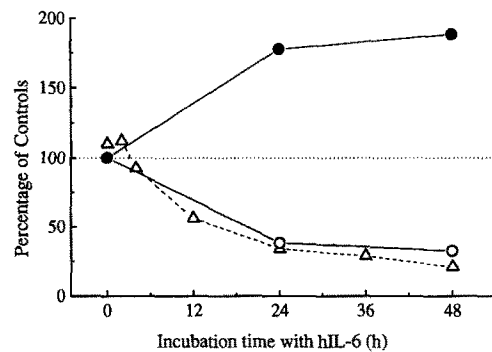


Figure 1. IL-6 induced time-dependent growth inhibition is by G1 cell cycle growth arrest. [3 H]thymidine incorporation (Δ) of WM35 human melanoma cells was determined after culturing the cells in the absence or presence of human IL-6 (10 ng/ml) for the period of time as indicated before cells were pulse labeled. Percentage of cells in G1 phase (\bullet) and S phase (\circ) were analyzed as described in Materials and Methods. Controls without IL-6 treatment at each time point were considered as 100%.

the transition in responsiveness of melanoma cells to IL-6 during disease progression.

The function of expressed IL-6 receptors was also assessed by analyzing [125 I]IL-6 binding to the gp80 receptor by Scatchard analysis. As summarized in Table II and Fig. 3, there was no significant difference between IL-6-sensitive and IL-6-resistant groups in terms of [125 I]IL-6 binding affinities and binding sites per cell. The cell line 9- Δ 6-1 used in this experiment was cloned from WM9 after being transfected with an IL-6 antisense expression vector. WM9 cells normally produce IL-6 (see below) and we wished to isolate variants of WM9 cells which were significantly suppressed in a constitutive manner for IL-6 production to test in vivo (our own unpublished observations). The 9- Δ 6-1 cells are resistant to IL-6 mediated growth inhibition. In Scatchard analysis, we used the CESS cell line as a positive control and obtained a binding affinity and receptor number comparable with those published by Taga et al. (51).

Endogenous IL-6 Production by IL-6 Resistant Cell Lines

Northern blotting analysis of poly(A) $^+$ RNA from these human melanoma cell lines revealed that IL-6 mRNA was de-

Table I. Cell Cycle Analysis of Human Melanoma Cell Lines With or Without IL-6 Treatment*

IL-6 added	Percent G1 phase cells		Percent G2/M phase cells		Percent S phase cells	
	-	+	-	+	-	+
WM 35	52.2	74.7	11.6	5.0	36.2	20.3
WM 902B	67.4	87.3	13.4	8.5	19.3	4.2
WM 1341B	74.6	83.2	0.6	3.6	24.8	13.2
WM 75	63.1	84.4	6.3	2.0	30.6	16.5
WM 983A	65.2	64.2	1.9	5.1	32.9	30.7
SKMEL 28	75.3	73.3	3.5	3.5	21.3	23.2

* 1×10^6 cells were cultured in 100-mm dishes in RPMI 1640 medium containing 2% FBS with or without rhIL-6 at 20 ng/ml for 24 h. Nuclei preparations were used for cell cycle analysis after propidium iodide staining. Human melanoma cell lines WM 35, WM 902B, WM 1341B, and WM 75 are growth inhibited by IL-6, but WM 983A and SKMEL 28 are not (30).

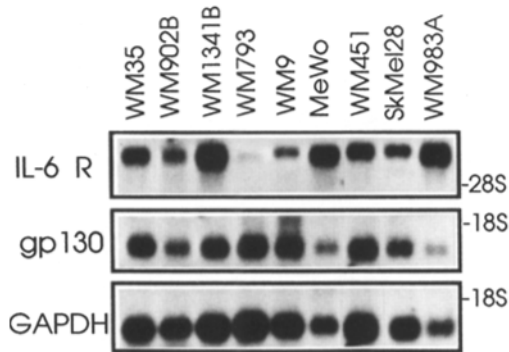


Figure 2. Northern blot analysis of IL-6 receptor and gp130 gene expression in human melanoma cell lines. 3 μ g of poly(A)⁺ RNA from each cell line was loaded into each lane before electrophoresis. cDNA probes for IL-6 receptor, gp130 and GAPDH used were as described in Materials and Methods.

detectable in five out of eight cell lines that are resistant to IL-6 growth inhibition. None of the three IL-6 sensitive cell lines showed evidence of endogenous IL-6 expression (Fig. 4). Similar IL-6 mRNA levels were found in another separate experiment. To address the question of whether these five cell lines—WM793, WM1205, WM9, MeWo, and WM1361A—produce and secrete biologically active IL-6, we tested the CM obtained from these cell cultures in the B9 murine cell bioassay. CM from three IL-6-sensitive cell lines did not demonstrate significant stimulatory activity for B9 cells, as monitored by [³H]thymidine incorporation. In contrast, CM from the five IL-6-resistant cell lines containing detectable levels of IL-6 mRNA were found to significantly stimulate the proliferation of B9 cells in both FBS containing CM1 (data not shown) and serum-free CM2 (Fig. 5 A). Moreover, the stimulatory activity on B9 cells by conditioned medium could be abrogated by addition of neutralizing anti-hIL-6 antibodies (Fig. 5 B), indicating that the stimulatory activity produced by melanoma cell is secreted IL-6. The results of the B9 cell bioassay are consistent with the observation of Northern blotting analyses; both provided evidence that these five IL-6 resistant cell lines are positive for endogenous IL-6 production, and secrete this product into their micro-environment. These results are in agreement with those of

Table II. Scatchard Analysis of IL-6 Receptor in Human Melanoma Cell Lines*

Cell line	[³ H]thymidine incorporation (5 ng/ml IL-6): (percent of controls)	kD ($\times 10^{-10}$ M)	Receptor sites per cell
CESS		3.55 \pm 0.39	2,211 \pm 105
WM 35	30.1 \pm 1.4	5.67 \pm 1.07	580 \pm 53
WM 1341B	30.7 \pm 0.1	1.51 \pm 0.10	365 \pm 54
WM 902B	29.2 \pm 0.6	2.26 \pm 0.14	382 \pm 82
WM 983A	95.8 \pm 4.8	1.48 \pm 0.23	212 \pm 38
WM 451	91.2 \pm 2.7	1.48 \pm 0.27	463 \pm 9
SKMEL 28	94.0 \pm 5.6	2.86 \pm 0.72	415 \pm 103
9- Δ 6-1	89.6 \pm 5.4	2.34 \pm 0.00	755 \pm 93

* Data are expressed as mean and SE. The CESS cell line was used as a positive control.

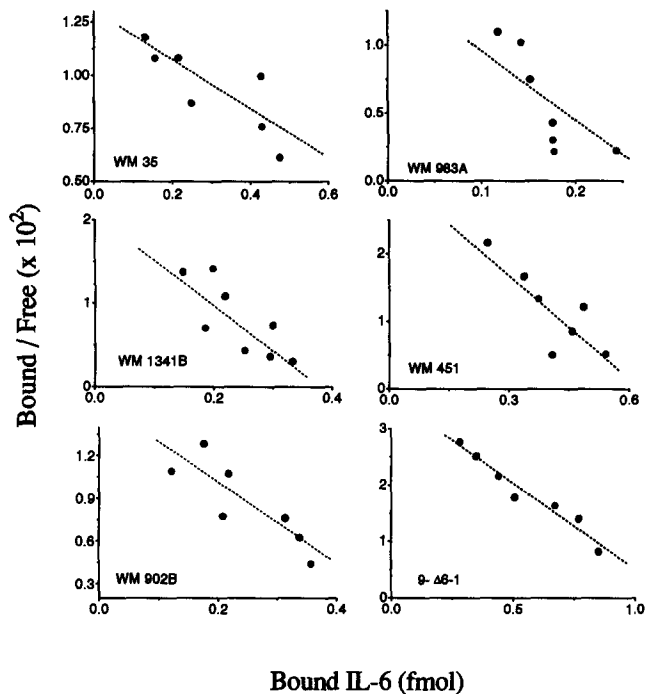


Figure 3. Scatchard plot analysis of [¹²⁵I]IL-6 binding to human melanoma cell lines. Representative data of Scatchard plot analysis from six human melanoma cell lines are shown. The three cell lines on the left are growth inhibited by IL-6, whereas the three on the right are not growth inhibited by exposure to exogenous IL-6. See also Table II.

Colombo et al. (7) who found that ~50–60% of advanced-stage human melanomas produce IL-6 in vitro.

Endogenous IL-6 Functions as an Autocrine Growth Stimulator for Melanoma Cells

Since we have shown that IL-6 is a potential growth inhibitor in human melanoma cell lines that are derived from early-stage (metastatically incompetent) primary lesions, the question we next addressed was the possible function of endogenous IL-6 in IL-6 resistant melanoma cell lines known to produce it. Initial in vitro analyses were undertaken by adding specific neutralizing anti-IL-6 antibodies into the cell culture medium. This did not result in any changes in [³H]-thymidine uptake in melanoma cells having endogenous IL-6

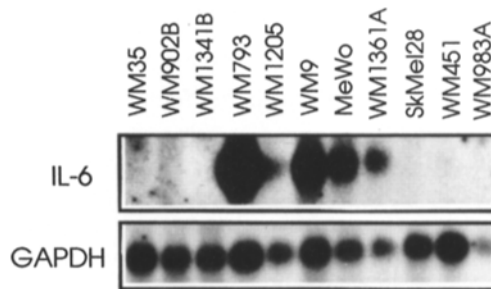


Figure 4. Northern blot analysis of IL-6 gene expression of human melanoma cell lines. 6 μ g of poly(A)⁺ RNA preparation from each cell line was used in each lane. Human IL-6 and GAPDH messages are both below 18S.

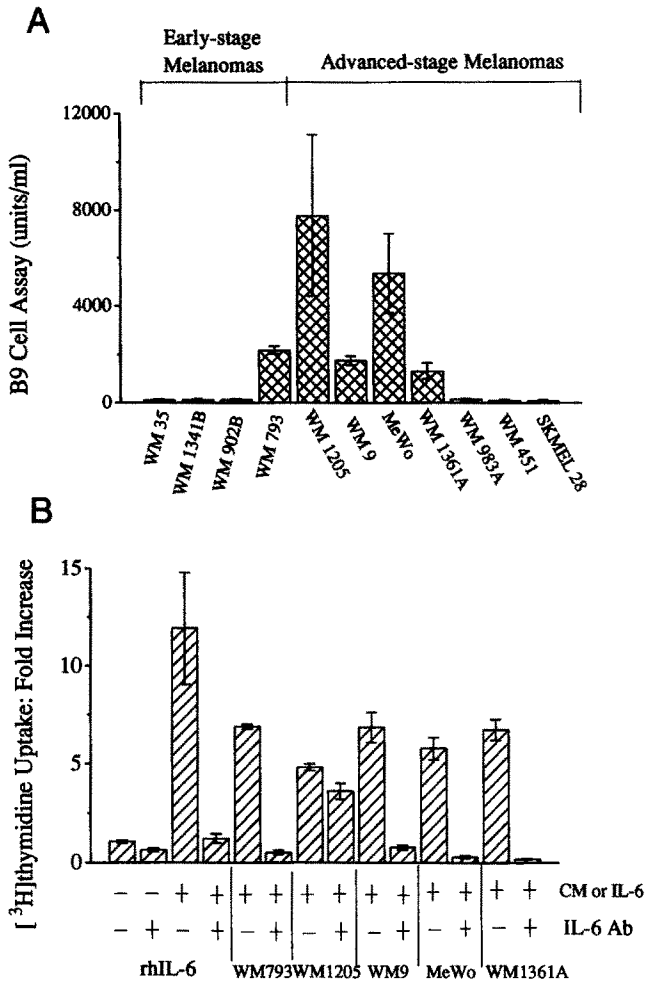


Figure 5. Stimulating activities on B9 cell proliferation by CM from human melanoma cell lines. (A) Serum-free CM2 from 11 human melanoma cell lines was assayed on B9 cells. Data are expressed by mean (\pm SE) of two separate experiments conducted in triplicate determination. (B) Addition of anti-hIL-6 neutralizing antibody (10 μ g/ml) was found to abrogate the stimulatory activity of either IL-6 (25 pg) or CM2 from melanoma cell lines for B9 cells.

production (Fig. 6). Failure of neutralizing antibodies to alter the growth in vitro, however, does not exclude the possible intracellular action of endogenous IL-6 in these cells, as previously described in other systems such as multiple myeloma (29). Antisense oligonucleotide to human IL-6 gene has been successfully used by others to down-regulate IL-6 production, a procedure that inhibited cell proliferation (29, 34). Therefore, we added a 15-mer antisense and a control sense oligonucleotide to human melanoma cell cultures. After 2 d incubation, addition of the antisense oligonucleotide into culture medium resulted in a significant inhibition in [3 H]thymidine incorporation in WM9 human melanoma cells compared to the control (sense) oligonucleotide (Fig. 7 A). A similar relative dose-dependent inhibition of [3 H]thymidine uptake was observed in both WM793 and MeWo cell lines (data not shown). When cell number was assessed, 10 μ M of oligonucleotide preparations were used in culture medium for 5 d. As shown in Fig. 7 B, cell proliferation was

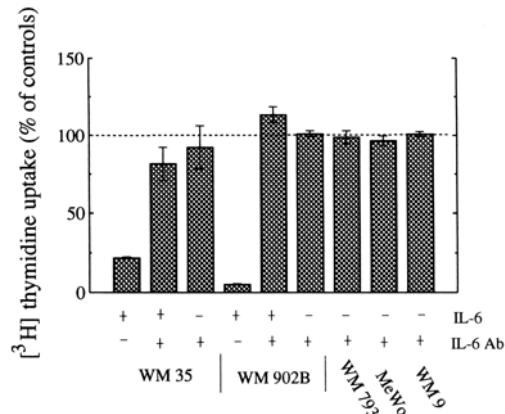


Figure 6. Addition of anti-IL-6 neutralizing antibody into culture medium does not affect the growth of melanoma cell lines that produce endogenous IL-6. Human melanoma cells (5×10^3 /well) were cultured in 96-well plates for 2 d with or without IL-6 neutralizing antibody (10 μ g/well). Human recombinant IL-6 (12.5 ng/ml) alone, or with IL-6 Ab, was added to WM35 and WM902B cell culture to show that the IL-6Ab is biologically active.

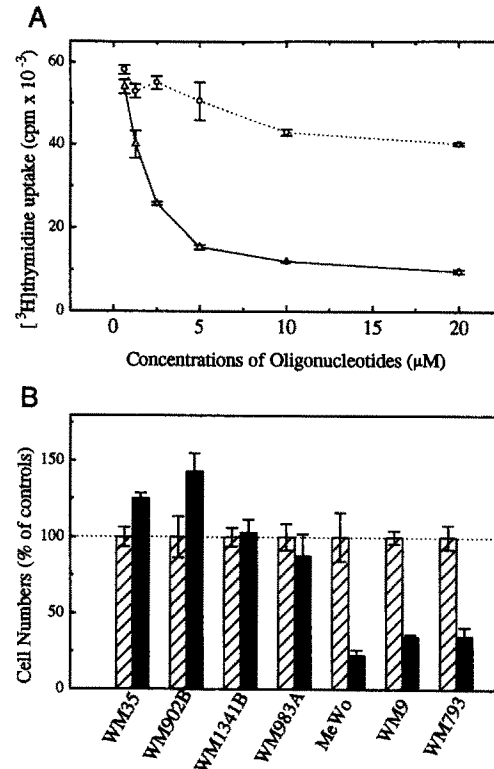


Figure 7. Effect of IL-6 antisense oligonucleotides to IL-6 on the growth of human melanoma cells. (A) WM9 human melanoma cells were plated in 96-well plates and incubated with different concentrations of sense (dotted line) and antisense (solid line) phosphorothioate oligonucleotides to the human IL-6 gene for 2 d. Cells were harvested 5 h after pulse labeling with [3 H]thymidine. (B) Antisense oligonucleotide (15-mer) preparation was added into culture medium (10 μ M) and cell numbers were counted 5 d after. Cell numbers in controls without addition of antisense oligonucleotide were considered as 100%. Similar results were obtained in a separate experiment. Data are expressed by mean (\pm SE) from a representative experiment.

significantly inhibited in all IL-6 producing melanoma cell lines. These results suggest a possible intracellular ("private") autocrine action of growth stimulation by IL-6 in human melanoma cells which produce this cytokine, similar to human multiple myeloma (29), although further studies are required to verify this possibility. The failure to achieve total inhibition of cell growth may be related to incomplete inhibition of the antisense oligonucleotide or to the production of additional autocrine growth factors by human melanoma cells such as bFGF and melanocyte growth stimulating activity, i.e., MGSA (26).

Discussion

In recent studies we found a selective growth inhibition of human melanoma cell lines that were derived from patients with early-stage benign (curable) lesions, whereas all seven cell lines obtained from advanced-stage (metastatically competent) lesions were resistant to IL-6 growth inhibition (30). The results of the present studies do not support the notion that differential [¹²⁵I]IL-6 binding to its receptors between IL-6-sensitive and resistant cell lines as being the mechanism mediating the selective inhibition. Northern blotting analysis also revealed similar mRNA levels for both IL-6 receptor and its associated IL-6 signal transducing protein, gp130. Thus, although the mechanism of the resistant phenotype to IL-6 induced growth inhibition in advanced-stage human melanomas remains to be clarified, in all likelihood it does not involve defective or altered cell surface IL-6 receptors.

Endogenous IL-6 production was assessed by both Northern blotting analysis and the B9 cell proliferation bioassay. Surprisingly, five out of eight melanoma cell lines known to be resistant to IL-6-mediated growth inhibition were found to be positive for endogenous IL-6 production. This prompted us to ask whether this endogenous IL-6 had any function on melanoma cell growth. There are at least three possibilities: (a) IL-6 could still function as a growth inhibitor but in this case the melanoma cells are sufficiently tolerant to overcome the inhibition; a precedent for this is found in TGF- β in which we previously showed that a colon carcinoma cell line was growth stimulated *in vivo* when transfected with an antisense TGF- β 1 expression vector (61); (b) it may be inert functionally as found in three other IL-6 resistant cell lines, which are negative for endogenous IL-6 production; or (c) it may act as a growth stimulator by an autocrine mechanism as has been found in some hematologic cancers (24, 34, 29, 40, 65). To analyze this problem, we added neutralizing antibodies to IL-6 into cell culture medium but this failed to alter the *in vitro* growth of melanoma cell lines endogenously positive for IL-6 production. However, the consistent growth inhibition we detected by addition of antisense oligonucleotides to the IL-6 gene into cell culture medium clearly indicated that endogenous IL-6 functions as a growth stimulator in these human melanoma cell lines, in all likelihood at an intracellular level. More recently we have found that transfection of an IL-6 antisense expression vector into IL-6 positive WM9 and MeWo melanoma cell lines significantly reduced their growth in nude mice (our own unpublished observations). Thus, when taken together with previous observations (30, 49) our results strongly suggest that IL-6 acts as a "bifunctional" cytokine for human melanoma cells during malignant tumor progression: it acts as a growth inhibi-

tor for normal melanocyte (49) and for early-stage primary melanomas by a paracrine mechanism (30) whereas the more advanced-stage form of this malignancy has a distinct tendency to become resistant to IL-6 induced growth inhibition—and in some cases to use it endogenously as a means of stimulating their own growth. Tumor cell-associated IL-6 could also affect tumor growth *in vivo* by additional mechanisms, for example by influencing tumor angiogenesis (37) and/or by affecting the ability of the immune system to recognize and destroy (immunogenic) tumor cells (41).

Acquisition of endogenous IL-6 production and its possible contribution in autocrine cell growth have been implicated as a key step in certain types of hematologic malignant tumor progression (21, 24, 29, 40, 65). However, the effects and function of IL-6 in human malignancies of non-hematopoietic origin would appear to be more complicated. For example, endogenous expression of IL-6 has been found in prostate and ovarian carcinoma cells as well as glioblastoma cells with no indication as yet about its function on the growth or other properties of these cancers (47, 56, 60). Growth inhibition or stimulation mediated by IL-6 were reported for human breast carcinoma (5, 39) and renal cell carcinoma, respectively (33, 52). Moreover, in a recent study, no significant effect of human IL-6 on the *in vitro* growth of more than 26 human malignant nonhematopoietic cell lines, including two cell lines of breast carcinoma was observed (46). Since the latter study involved the use of advanced-stage malignant tumors, exogenous IL-6 might not be expected to have an effect, consistent with our melanoma results. However, many of these tumors may be inhibited by IL-6 in an earlier stage of tumor progression, and conversely, may be stimulated "cryptically" in more advanced stages by endogenously produced IL-6. Of possible significance in this regard, Sehgal and his associates have reported that the wild-type p53 suppressor gene may normally suppress IL-6 gene expression in epithelial cells (43). Hence, inactivation of the p53 gene by deletion and/or mutation may lead to ectopic production of IL-6 in carcinomas and other types of tumors which undergo such genetic alterations. This may help explain the high frequency of IL-6 production *in vitro* (see above) or *in vivo* (43, 50) by solid tumors, since p53 gene mutations are found in as many as 50% or more of all cancers (12). While our results have concentrated on IL-6 as a possible hemopoietic autocrine growth factor in nonhematologic malignancies, it should be noted that other types of hemopoietic growth factors have been implicated as possible autocrine stimulators of certain carcinomas. For example, CSF-1 has been implicated to function in such a manner in both human breast and ovarian cancers (22, 23, 53).

Progressive resistance or 'emancipation' (28) to the action of inhibitory growth factors during tumor progression has been noted in the context of other growth factors, especially TGF- β (20, 31, 32, 36, 44, 54, 63) as reviewed recently by Kerbel (26). Furthermore, in some cases paracrine TGF- β has been shown to change from acting as an inhibitor to a growth stimulator during transition from low grade to well differentiated high grade advanced malignant cancers (19, 20, 44, 63). However, to our knowledge, there is no precedent for a growth factor functioning as a potential paracrine growth inhibitor during one stage of tumor progression, and as an autocrine growth stimulator at a later, more malignant stage, as shown in this report. Such transitions in responsive-

ness may contribute to the ability of malignant (metastatically competent) tumor cells to grow progressively at the primary tumor site, i.e., to manifest a "growth dominant" phenotype (25), as well as to grow in distant organ sites. Our results have two other important potential implications. First, they imply that IL-6—and by implication perhaps other members of the hematopoietic growth factor family—may contribute to the growth of solid tumors in a more significant way than previously suspected. Second, they raise the intriguing possibility of "multi-cytokine resistance" (e.g., to TGF- β and IL-6) and multi-growth factor independence (11) being important aspects of the growth of advanced stage, metastatic, cancers (26).

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