

# Increased Resistance to Oncostatin M-induced Growth Inhibition of Human Melanoma Cell Lines Derived from Advanced-Stage Lesions<sup>1</sup>

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## Abstract

Human melanomas can become progressively resistant to the growth-inhibitory effects of a broad family of structurally diverse cytokines which includes interleukin 6 (IL-6). Uncovering this multicytokine resistance was made possible by the availability of cell lines established from early-stage radial growth phase or vertical growth phase primary melanomas as well as more advanced primary lesions and distant metastases. Because Oncostatin M (OSM) is also a member of the IL-6 family we evaluated the effects of this cytokine on the growth of human melanoma cell lines obtained from different stages of disease progression. The results showed that three different cell lines derived from early-stage melanomas were strongly growth inhibited by OSM, as they are by IL-6. Three cell lines, established from advanced-stage melanomas, were growth inhibited by OSM, but much higher concentrations (in the range of 10-fold) were required to obtain 50% growth inhibition; these cell lines were not inhibited by IL-6. Three other cell lines that were IL-6 resistant (two of which were advanced stage) were also found to be OSM resistant. Only one advanced-stage IL-6-resistant cell line was found to be highly sensitive to OSM-mediated growth inhibition. In addition, we found that variants isolated from early-stage WM35 melanoma cells that possess a much more aggressive tumorigenic phenotype in nude mice were significantly more resistant to both OSM- and IL-6-mediated growth inhibition. The results demonstrate that OSM can function as a growth inhibitor of human melanoma cells but that its ability to do so is progressively diminished or lost with disease progression. This finding is consistent with the concept of acquired "multicytokine resistance" during melanoma progression.

## Introduction

The growth advantage of cancer cells over their normal cellular counterparts is thought to be due in part to the acquisition of relative resistance to certain autocrine or paracrine growth-inhibitory cytokines (1). Foremost among these are members of the TGF- $\beta$  family, such as TGF- $\beta_1$  and TGF- $\beta_2$ . Whereas normal epithelial cells from a variety of organs are strongly inhibited by exposure to low concentrations of TGF- $\beta$ , corresponding carcinomas derived from these epithelial cells are frequently resistant to this inhibition (1). The same is generally true for leukemias and lymphomas (1).

More recent studies have shown that resistance to TGF- $\beta$  is frequently progressive, *i.e.*, the more biologically advanced and aggressive a lesion, the greater the degree of resistance its cells have to TGF- $\beta$ -mediated growth inhibition (2). This has been shown in human cancers such as skin tumors (3), glioblastomas (4), and colorectal carcinomas (5–7). We have also observed progressive TGF- $\beta$  resis-

tance in human malignant melanomas (8). Thus cell lines established from early-stage RGP or "thin" VGP primary melanomas are growth inhibited by exposure to TGF- $\beta_1$  (as are normal melanocytes), whereas most cell lines established from more advanced VGP primary melanomas or melanoma metastases are resistant or partially resistant to this inhibition (8). A similar pattern has been observed when studying more aggressive variants isolated from early-stage human melanomas.<sup>4</sup>

The relative resistance of advanced-stage metastatically competent melanomas to TGF- $\beta$  is also accompanied by a similar relative resistance to other, structurally unrelated cytokines. These include IL-6, IL-1, and tumor necrosis factor  $\alpha$ , as recently described by us (9, 10). We have coined the term "multicytokine resistance" to describe this phenomenon (2); it may be a major factor in endowing melanoma cells with an ability to grow in the foreign environment of the dermal mesenchyme (2) and achieving "clonal dominance" of metastatically competent cells within primary tumors (11).

The multicytokine resistance phenomenon and the finding that it includes IL-6 prompted us to determine whether another "hemopoietic" cytokine (OSM) can behave as an inhibitor for early-stage human melanomas and whether its ability to do so is lost in advanced-stage lesions. OSM is a glycoprotein that was originally isolated from the conditioned medium of a human histiocytic leukemia cell line (U937) by its ability to inhibit DNA synthesis of A375 human melanoma cells (12, 13). The complementary DNA sequence of OSM, which was cloned shortly thereafter, revealed that OSM is one of the members of the IL-6 family of cytokines (14, 15), of which there are several members (15, 16). There is a close relationship among IL-6, OSM, and LIF in terms of ligand-binding and signal-transducing activities (17–19). It also appears that OSM can mimic most of the biological activities of IL-6 (20–22).

The main purpose of this report was to test the effects of OSM on the growth of a large panel of human melanoma cell lines established from various stages of disease progression. Evidence was obtained to show that its capacity to act as an inhibitor does indeed diminish with tumor progression, making it similar to IL-6 and certain other cytokines.

## Materials and Methods

**Cell Lines and Other Reagents.** Human melanoma cell lines of the WM series were obtained from Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA). The origins and establishment of these cell lines have been described previously (23, 24). The WM 35 cell line was derived from RGP lesion, and WM 902B, WM 1341B, and WM 793 were derived from "thin" VGP primary lesions. Patients were cured after surgical removal of these tumors, and therefore the cell lines were considered as "early-stage" and metastatically incompetent. Advanced-stage melanoma cells were from either primary tumor of thick VGP lesions (*e.g.*, WM 983A and WM 1361A) or distant metastases (WM 9, WM 451). The MeWo and SKMEL 28 cell lines had been described

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<sup>3</sup> The abbreviations used are: TGF, transforming growth factor; IL, interleukin; RGP, radial growth phase; VGP, vertical growth phase; OSM, oncostatin M; ID<sub>50</sub>, concentration causing 50% inhibition; LIF, leukemia inhibitory factor.

<sup>4</sup> H. Kobayashi, S. Man, J. R. MacDougall, C. H. Graham, C. Lu, and R. S. Kerbel. Development of "multicytokine resistance" during human melanoma progression: analysis using tumorigenic variants of low-grade early-stage human melanomas, submitted for publication.

Table 1 Summary of the effect of OSM on [<sup>3</sup>H]thymidine incorporation in human melanoma cell lines derived from different stages of tumor progression<sup>a</sup>

Cell Line	Origin	Response to OSM	ID <sub>50</sub> (ng/ml) <sup>b</sup>	ID <sub>50</sub> (ng/ml) (to IL-6) <sup>c</sup>
Early-stage melanoma cell lines				
WM 35	Early-stage RGP (primary)	↓↓ <sup>d</sup>	16.4	1.5
WM 902B	Early-stage VGP (primary)	↓↓	32.3	1.0
WM 1341B	Early-stage VGP (primary)	↓↓	7.2	14.0
WM 793	Early-stage VGP (primary)	NR		NR
Advanced-stage melanoma cell lines				
WM 983A	Advanced-stage VGP (primary)	NR		NR
WM 1361A	Advanced-stage VGP (primary)	NR		NR
WM 9	Metastasis	↓	376.4	NR
WM 451	Metastasis	↓	459.0	NR
MeWo	Metastasis	↓↓	48.9	NR
SKMEL 28	Metastasis	↓	223.6	NR

<sup>a</sup> [<sup>3</sup>H]Thymidine incorporation was tested in 96-well plates in the absence or presence of human recombinant OSM. Data were obtained from two to six separate experiments performed by triplicate determinations.

<sup>b</sup> ID<sub>50</sub> was calculated by the dose-dependent inhibition at 50% in DNA synthesis and is expressed as ng/ml of OSM. ID<sub>50</sub> for WM 9, WM 451, and SKMEL 28 was obtained by testing the responses to OSM up to 500 ng/ml; NR, no response (no stimulation, no inhibition).

<sup>c</sup> Growth response to exogenously added IL-6 in culture was cited from Ref. 9.

<sup>d</sup> ↓↓ and ↓, growth inhibition of cells of more than and less than 50%, respectively, by OSM in the concentration ranges from 0.1 to 100 ng/ml, compared to controls (considered as 100%).

previously (24). The A375 cell line was established in 1973 with no details regarding its pathologic classification (25). Several variants of WM 35 cell line (P1P, P2P, and P3P) were isolated after *in vivo* passage and selection in nude mice using "Matrigel assistance" methods.<sup>4</sup> These cell lines, derived from tumors in nude mice, were shown to be of human origin without the contamination of mouse cells.<sup>4</sup> P1P, P2P, and P3P were referred to as the pooled populations of *in vivo* passages 1, 2, and 3, respectively, as described.<sup>4</sup> The selection of these sublines was based on the fact that WM 35 is poorly tumorigenic in nude mice but that it will grow readily when injected along with Matrigel.<sup>4</sup> The tumors which arose were adapted to culture. The cells from such sublines were then found to be highly tumorigenic in nude mice in the absence of Matrigel.<sup>4</sup>

Recombinant human IL-6 was purchased from Upstate Biotechnical, Inc. (Lake Placid, NY). Recombinant human OSM was purchased from Peppo Tech, Inc. (Rocky Hill, NJ) with an ED<sub>50</sub> of 2 ng/ml in TF-1 cells. Recombinant human LIF was purchased from R & D Systems (Minneapolis) with biological activity (ED<sub>50</sub>) of 0.15 to 0.3 ng/ml in TF-1 cells. ExCell 300 medium was purchased from J. R. Scientific (Woodland, CA).

**Cell Culture and [<sup>3</sup>H]Thymidine Incorporation Assay.** Cell culture and [<sup>3</sup>H]thymidine incorporation assay (to measure cell growth) were conducted as described previously (9, 24). Human melanoma cells were maintained in RPMI containing 5% fetal bovine serum. The growth assay was performed in ExCell 300 medium containing 1% fetal bovine serum and various concentrations of cytokine in 96-well plates in a final volume of 150 μl. After 2 days of incubation with cytokine, cells were pulse labeled with [<sup>3</sup>H]thymidine for 4 to 6 h before being harvested into Printed Filtermat A in a Titertek Cell Harvester 530. The residual radioactivity on filter was counted in a 1205 Betaplate scintillation counter (Wallac, Gaithersburg, MD). The radioactivity in the wells tested was calculated against control wells without cytokine treatment, which were considered as 100%. ID<sub>50</sub> was calculated by the concentration of cytokine that caused 50% inhibition of [<sup>3</sup>H]thymidine incorporation and expressed by the mean value from two to six separate experiments that were performed in triplicate determinations. Cell growth was evaluated using [<sup>3</sup>H]thymidine incorporation, rather than direct cell counts, since previous studies have shown that the thymidine incorporation assay reflects changes in cell numbers when using the human melanoma cell lines described in this study (9, 10, 24). Therefore we decided to monitor cell growth using the simpler and shorter thymidine incorporation assay.

## Results

**Increased Resistance to OSM-induced Growth Inhibition in Advanced-Stage Human Melanomas.** The effect of OSM on human melanoma cell lines derived from different clinical stages of disease progression was tested in ten different cell lines, as listed in Table 1. To compare the response to OSM, the effect of IL-6 on these cells was cited from our previous work (9). As summarized in Table 1, three of four cell lines that were derived from early-stage melanomas were

growth inhibited by the addition of recombinant OSM, which is the same as what was observed after IL-6 exposure. However, there is a difference in the sensitivities to the growth inhibition induced by IL-6 or OSM in terms of ID<sub>50</sub> values. WM 902B is 4.5 times less sensitive to OSM but 10 times more sensitive to IL-6 than is WM 1341B, both of which were derived from early VGP lesions (9). The exception in this category of early-stage melanoma is the WM793 cell line; it was not growth inhibited by OSM, which is in accordance with its resistance to IL-6 (9).

Among cell lines that were derived from advanced-stage melanomas, two did not respond to exogenously added OSM (WM 983A and WM 1361A), which is in agreement with our previous IL-6 results (9). However, three cell lines (WM 9, WM 451, and SKMEL 28) manifested some responsiveness, albeit much less than WM 35, WM 1341B, and WM902B, to OSM-induced growth inhibition (despite the fact that they are not known to be growth inhibited by the addition of IL-6). Thus, as summarized in Table 1, the ID<sub>50</sub> values were in the range of 223 to 459 ng/ml, whereas ID<sub>50</sub> values in the range of 7.2 to 32 ng/ml were obtained with the three early-stage lines that were inhibitable. Only the MeWo cell line had a comparable ID<sub>50</sub> value, among the advanced stage cell lines tested.

**Differential Effect of OSM on Human Melanoma Cell Lines with IL-6 and LIF.** The discrepant responses to IL-6 and to OSM of some human melanomas were found in four of the six cell lines from advanced-stage lesions (Table 1). One example is the MeWo cell line. MeWo was growth inhibited by OSM at relatively low concentrations but not by the addition of exogenous IL-6 (Fig. 1A). Previous work has shown that IL-6 can act as an autocrine growth stimulator for MeWo cells (10).

Recent studies have shown that OSM may share a receptor with LIF (19), and both use gp130 as their receptor and/or signal transducer (17, 18). However, when the effect of LIF on human melanomas was tested, there was no significant growth inhibition or stimulation of any of the melanoma cell lines tested, such as WM 35, WM 1341B, WM 902B, WM 9, and WM 451 (Fig. 1B).<sup>5</sup> Besides LIF, the possible involvement of granulocyte colony-stimulating factor in the growth regulation of human melanomas was also tested, but we were unable to observe any effect of granulocyte colony-stimulating factor on DNA synthesis *in vitro* in any of the melanoma cell lines tested.<sup>5</sup>

**Additive Effect of IL-6 and OSM on the Growth Inhibition in Human Melanoma Cells.** The combined effect of IL-6 and OSM was investigated by use of A375 melanoma cell line. As shown in Fig.

<sup>5</sup> Unpublished observations.

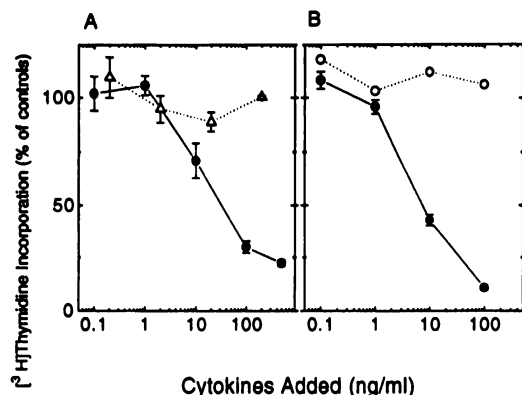


Fig. 1. Differential response of  $[^3\text{H}]$ thymidine incorporation by exposure to IL-6, LIF, and OSM.  $[^3\text{H}]$ thymidine incorporation was tested to estimate cell growth in response to IL-6, LIF, and OSM. Data are expressed as the mean and SE from one representative experiment performed by triplicate determinations. Cells without cytokine treatment were considered as controls (100%). A, response of MeWo cells to IL-6 ( $\Delta$ ) to OSM ( $\bullet$ ). B, response of WM 1341B cells to LIF to ( $\circ$ ) and to OSM ( $\bullet$ ).

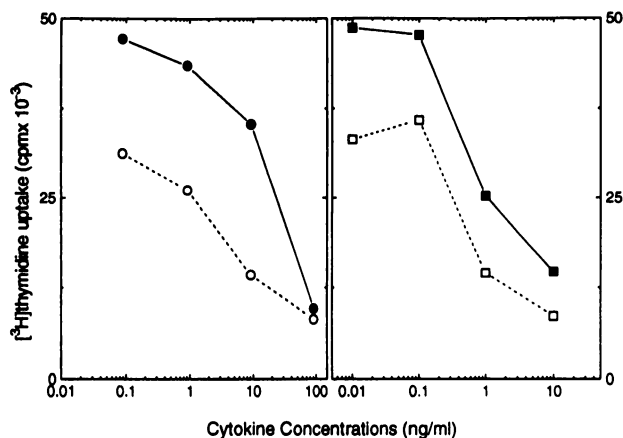


Fig. 2. Additive effect of IL-6 and OSM in inhibiting cell growth in human A375 melanoma cells.  $[^3\text{H}]$ thymidine incorporation was measured in the presence or absence of IL-6 or OSM. Left, increased amount of OSM without ( $\bullet$ ) and with ( $\circ$ ) 1.0 ng/ml IL-6. Right, increased amount of IL-6 without ( $\blacksquare$ ) and with ( $\square$ ) 9.33 ng/ml OSM.

2, there is a clear additive effect of growth inhibition on A375 cells between IL-6 and OSM. A similar additive effect of IL-6 and OSM was also observed in another human melanoma cell line, WM 35, that was derived from a patient with a RGP lesion (data not shown). The growth of WM 35 cells is strongly inhibited by IL-1, IL-6, and tumor necrosis factor  $\alpha$  (9), as well as TGF- $\beta$  (8).

**Increased Resistance to OSM-induced Growth Inhibition in an Experimentally Developed Human Melanoma Progression System.** An alternative method of evaluating whether OSM loses its ability to inhibit the growth of human melanoma cells with disease progression would be to test variants of early-stage melanomas that manifest a significantly more aggressive capacity for tumor growth *in vivo*. We have selected such variants from early-stage WM 35, WM 1341B, and WM 793 cells by injection of these poorly tumorigenic (WM 793 being an exception in this regard) cell lines into nude mice comixed with Matrigel, as a means of generating highly tumorigenic variants.<sup>4</sup> The process was serially repeated so as to generate a series of related variants having increasing tumorigenic properties; these were called "P1" (passage 1), "P2" (serial passage 2), and so forth. These variants were previously found to manifest increased resistance to TGF- $\beta_1$ -mediated growth inhibition (8) and to inhibition by IL-6, IL-1 $\alpha$ , and TNF- $\alpha$ .<sup>4</sup> As shown in Fig. 3, a similar increase in resistance to the inhibitory effects of OSM was also noted using *in vivo*-selected variants of WM 35 cells.

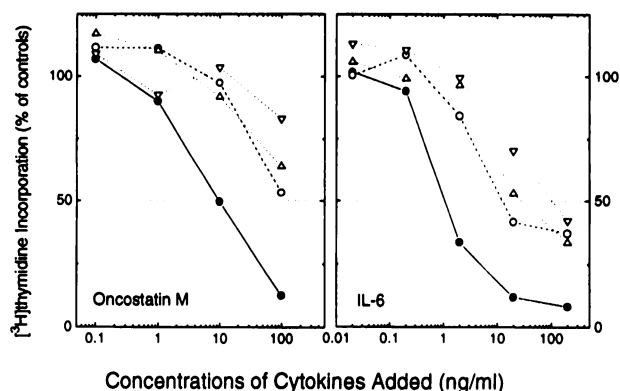


Fig. 3.  $[^3\text{H}]$ thymidine incorporation in response to OSM and IL-6 in the WM 35 cell line and its *in vivo* selected highly tumorigenic variants. Cell growth was tested by  $[^3\text{H}]$ thymidine incorporation in the presence or absence of OSM and IL-6. Data are expressed as the mean and SE of triplicate determinations from a representative experiment. Cell lines used were parental WM 35 ( $\bullet$ ) and the pooled population after *in vivo* passage. P1P ( $\circ$ ), P2P ( $\Delta$ ), and P3P ( $\nabla$ ) are sublines of WM 35 that were serially passaged one, two, and three times, respectively, in nude mice as described elsewhere. These sublines have a significantly enhanced ability to form tumors in nude mice in comparison to the weakly tumorigenic WM 35 parental cell line.<sup>4</sup>

## Discussion

Oncostatin M was originally purified and characterized as a cytokine with growth-inhibitory properties for the human melanoma cell line called A375 (12, 13). The results of our studies confirm the conclusion that OSM can inhibit the growth *in vitro* of human melanoma cells, but with one important qualification: that this property is frequently attenuated, or lost altogether, as melanomas progress. Thus, three of four independent early-stage melanoma cell lines were found to be strongly growth inhibited by exogenous OSM, whereas only one of seven advanced-stage melanoma cell lines was comparably inhibited. Of the other six cell lines, three were OSM resistant (in terms of growth inhibition) and three were inhibitable; however, much higher concentrations of OSM were required to achieve 50% inhibition of cell growth in these three cell lines (as measured by thymidine incorporation) in comparison to the three inhibitable early-stage cell lines. Moreover, when genetically related variants of early-stage poorly tumorigenic WM 35 cells, selected for high grade tumorigenicity in nude mice,<sup>4</sup> were examined, they manifested a significant degree of resistance to OSM-mediated growth inhibition. Taken together, the results show that OSM can be added to the list of cytokines known to be strong inhibitors of most cell lines established from early-stage melanomas but whose capacity to bring about such growth inhibition is diminished or lost with melanoma progression (8–10).<sup>4</sup> This list includes TGF- $\beta_1$ , IL-6, IL-1 $\alpha$ , IL-1 $\beta$ , and tumor necrosis factor  $\alpha$  (8, 9). The significance of OSM to this "multicytokine resistance" (2) remains to be determined as well as the phenomenon itself in terms of its contribution to tumor growth and metastatic spread *in vivo*.

The possible mechanisms that could account for the differential response to OSM and IL-6 in the melanoma cell lines we analyzed are unclear. The genes for receptors for IL-6 have been cloned and are known to be composed of two components, gp80 with high binding activity for IL-6, and gp130, which has no IL-6 binding activity but which mediates IL-6 signal transduction upon IL-6 binding to gp80 (26). The structure of OSM receptor complexes is poorly understood. It is known that OSM can bind to both gp130 and the LIF receptor (17–19, 26). Our previous work indicated that the differential response to IL-6 was not due to differences in gene expression of gp80 and gp130 nor to IL-6 ligand binding activity (10). Whether this is the case for OSM has yet to be determined in terms of ligand binding activity.

The nonresponsiveness of our melanoma cells to LIF supports a previous report showing differential activity of LIF comparing to

OSM and IL-6 on A375 cells even though IL-6, LIF, and OSM are all effective in the terminal growth arrest and differentiation control in myeloblastic M1 murine leukemia cells (20). Since there is a strong discrepancy in the response to IL-6, OSM, and LIF in some of the advanced-stage melanoma cell lines, we suggest that there are distinct signal mechanisms among IL-6, OSM, and LIF in addition to their common pathway, *i.e.*, there may be both "private" and "public" (gp130) pathways of action, at least within the melanocytic cell lineage.

In summary, our results show yet another member of the hemopoietic family of cytokines, Oncostatin M, can function as a strong paracrine growth inhibitor for human melanoma cells, provided the cells are derived from early-stage primary tumors. The capacity to inhibit is weakened in most of the advanced-stage lesions that were examined. OSM is known to be made by macrophages and T-lymphocytes, cells which can be found in the dermis and are associated with growing primary melanomas (27). Hence, there is the distinct possibility that OSM may help contribute to the growth advantage of small numbers of metastatically competent melanoma cell variants residing within early-stage primary melanomas *in vivo*. This may assist such variants in overgrowing their less malignant counterparts and help to achieve "clonal dominance" in more advanced primary melanomas (2, 11).

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