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Cytokine Sensitivity of Metastatic Human Melanoma Cell Lines – Simultaneous Inhibition of Proliferation and Enhancement of Gelatinase Activity

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The effect of a panel of cytokines on the proliferation and type IV collagenase production was studied in four melanoma cell lines of different origin, tumorigenicity and metastatic capacity. TGF-β, TNF-α and to a lesser extent, IL-1α exhibited antiproliferative effect on the cell lines, with some lines showing varying degree of resistance. The sensitivity did not correlate directly with the origin or the biological behavior of the tumor lines, suggesting that cytokine resistance of advanced stage melanoma cells may be relative. IL-2, IL-10 and IL-12 displayed little or no effect on proliferation. The effect of cytokines on metalloproteinase production showed a cell line dependent pattern. Interestingly, those cytokines that exhibited the most pronounced antiproliferative activity, also proved most effective in stimulating collagenase secretion, often simultaneously, in the same line. The results indicate that pleiotropic cytokines can have positive and negative effects simultaneously on various steps of tumor progression. (Pathology Oncology Research Vol 4, No 2, 108–114, 1998)

Key words: melanoma, cytokines, proliferation, metalloproteinases

Introduction

Metastasis is a complex process involving a series of interactions between the tumor cells and their environment. Cytokines, secreted either by the tumor cells or by host cells, represent one of the important mediators of these interactions. Most of them can exert pleiotropic effects modulating different steps of the progression of malignant tumors.

Many cytokines have been shown to have growth inhibitory effects on various tumor cell types. These include TNF-α, TGF-β, IL-1, IL-6, interferons and others.[1-3] On the other hand, resistance to these factors can develop during tumor progression,[4-6] and in some cases the growth stimulatory activity of these and other cytokines has been described.[7,8]

Growth regulation is only one way in which cytokines modulate tumor progression. Many of these factors can also have an effect on multiple steps of the metastatic cascade. The inflammatory cytokines IL-1 and TNF-α have been shown to enhance metastasis formation by experimental tumors via stimulation of the expression of cell adhesion molecules on endothelial cells, thus stimulating endothelium – tumor cell adhesion.[9,10] Several cytokines can influence the expression of cell adhesion molecules on tumor cells,[11,12] or promote or suppress angiogenesis.[1,13,14]

The metastatic process involves several steps that require degradation of extracellular matrix proteins. Destruction of the subendothelial basement membrane is a prerequisite for dissemination of most cancer cells. Gelatinases, members of a family of zinc-dependent endopeptidases, the matrix metalloproteinases (MMP) are thought to play a major role in the digestion of one of the components of basement membrane, type IV collagen. Not surprising-

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ly, metastatic cancer cells have been shown to produce elevated amounts of various proteolytic enzymes, including MMP-2 (72 kDa type IV collagenase) and MMP-9 (92 kDa type IV collagenase).[15,16]

The regulation of MMP-2 and MMP-9 have distinct characteristics. The human MMP-9 gene promoter contains consensus AP-1, Sp-1 and NF-κB sites,[17,18] that have been shown to mediate the induction by TPA, TNF-α and IL-1 in several tumor cell lines.[19,20] In contrast, the MMP-2 gene regulatory region lacks AP-1 and NF-κB binding sites,[21] and in most cell types upregulation of this enzyme has not been demonstrated after the exposure to TNF-α or IL-1.[19,20,22] A few other cytokines have been shown to influence the production of type IV collagenases, the mechanisms of which is not yet clarified. TGF-β stimulated MMP-2 and/or MMP-9 in certain melanoma, fibrosarcoma and carcinoma cell lines.[22-24] IL-8 has been shown to increase MMP-2 production in melanoma,[25] while IL-4 and IL-10 were inhibitory in this respect on prostate carcinoma cells.[26]

In this study, the effect of a panel of cytokines was studied on the proliferation and gelatinase secretion of four melanoma cell lines with varying tumorigenicity and metastatic potential. The effect of cytokines on proliferation and metalloproteinase production showed a cell line dependent pattern. TGF-β and TNF-α, two cytokines that exhibited the most pronounced antiproliferative activity, also proved the most effective in stimulating collagenase secretion of the same lines, indicating that cytokines can exert positive and negative effects simultaneously on the outcome of the malignant disease.

Materials and Methods

Cell lines and culture conditions

The HT168-M1 melanoma line is a derivative of the A2058 cell line, selected for high liver colonizing capacity in immunosuppressed CBA/Ca mice.[27] The A2058 melanoma cell line, provided by LA Liotta (NCI, Bethesda, MD) was derived from a brain metastasis.[28] The M24met line, kindly provided by B.M. Mueller (Scripps Research Institute, La Jolla, CA)[29] was established from a metastatic nodule of a nude mouse injected with M24 cells derived from a lymph node metastasis.[30] The WM983B line, derived from a metastasis, and WM35, derived from an early stage primary lesion[31] were gifts from M. Herlyn (Wistar Institute, Philadelphia, PA). The cell lines were maintained in RPMI 1640 medium (Sigma, St. Louis, USA) supplemented with 5% fetal bovine serum (Sigma), 0.1 mM non-essential amino acids (Gibco BRL, Life Technologies, Paisley, Scotland), 1 mM sodium pyruvate (Gibco), 2 mM glutamine and 50 μg/ml gentamycin sulfate (Gibco) at 37°C in a 5% CO_2 atmosphere.

Intrasplenic injection of tumor cells

Single-cell suspensions from 0.02% EDTA-treated monolayer cultures were washed and diluted in Hank's balanced salt solution (HBSS). One million viable cells were inoculated in the spleens of SCID mice in a volume of 50 μl.[27] Recipients were killed when they became moribund or 10 weeks after tumor cell implantation.

Cytokines

TGF-β, TNF-α, IL-1α and IL-12 were purchased from R&D Systems (Minneapolis, MN), IL-10 was purchased from Endogen (Cambridge, MA), and IL-2 from DuPont Medical Products (Wilmington, DE). All cytokines were reconstituted and used according to the manufacturer's instructions.

Cytokine treatments

For the proliferation assays, 4 x 10^3 melanoma cells were allowed to adhere to flat-bottomed 96-well tissue culture plates (Greiner, Germany) overnight in serum-containing medium, then the medium was replaced with serum-free medium and various concentrations of the different cytokines were added. For each cytokine, the medium used for reconstitution was applied in the appropriate dilutions as controls. At the end of the 96-h incubation period, a colorimetric assay (MTT test) was performed. All tests were done with 3 to 5 parallel samples.

For the collagenase production tests, 10^5 cells were plated in 24-well tissue culture plates (Greiner). After adherence, the cell layers were washed in HBSS, and the different cytokines were added in 0.5 ml serum-free medium. Forty-eight hours later the conditioned media were centrifuged and the supernates were frozen until use for zymography. In some cases, the assays were run on 96-well plates as described at the proliferation test, and after collecting 100 μl of the conditioned media, a colorimetric assay (SRB) was performed to determine cell density.

Cell proliferation assays

The MTT and SRB tests were performed according to Martin and Clynes.[32] In the MTT test, at the end of the 96-h incubation period 0.5 mg/ml of the tetrazolium dye MTT (Sigma) was added to the wells. After 4 h incubation at 37°C, the medium was gently removed, the plates air-dried, and the formazan crystals formed in viable cells were dissolved in DMSO.

For the SRB assays, cells in the 96-well plates were fixed with 10% TCA for 1 h at 4°C, washed in tap water and dried. Sulforhodamine B (Sigma) was added (0.4% in 1% acetic acid) for 20 min, and after washing in 1% acetic

Table 1. **Characteristics of the human melanoma lines used: origin, tumorigenic and metastatic potential**

Cell line	*Source of cell line*	*Tumorigenicity (s.c.)*	*Tumor formation after i.s. injection*[d]	
			spleen tumor	*liver metastasis*
HT168-M1	metastasis	+[a]	+	+
M24met	metastasis	+[b]	+	+
WM983B	metastasis	+[c]	+	+
WM35	primary (early)	+/−[c]	+/−[e]	−

[a]Ladányi et al, 1990[27]
[b]spontaneous metastases after s.c. injection in nude or SCID mice (Mueller et al, 1991[29])
[c]Herlyn 1990[31], Bani et al, 1996[6]
[d]formation of primary tumor and metastases after i.s. injection of 10^6 cells in SCID mice
[e]small tumors >2 months after injection in 2/6 SCID mice

acid and drying, 10 mM Tris was added to the wells. In both assays, the adsorbance at 570 nm was measured with a Labsystems Multiskan MS microplate reader.

Gelatin zymography

The measurements of type IV collagenase activity were performed as described.[33] Briefly, 15 µl of the samples were mixed with 15 µl of 2x sample buffer, and applied on an 8% SDS-PAGE (Serva, Germany) gel containing 0.3 mg/ml gelatin (Sigma). After electrophoresis at 25 mA/gel for 45 min the gels were washed in 2.5% Triton-X-100 for 30 min and incubated overnight at 37°C in a solution containing 50 mM Tris-HCl (pH 7.6), 5 mM $CaCl_2$, 1 µM $ZnCl_2$ and 0.02% NaN_3. The gels were then stained with 0.2% Coomassie Brilliant Blue (Serva) in 50% methanol, followed by destaining in 20% methanol and 10% acetic acid. Areas of gelatinolytic activity appeared as bands of clearing on the gels. Enzyme activities were quantitated by Eagle Eye II video density system (Stratagene, Germany). The OD values were corrected with the cell densities given as ODs measured in SRB tests at the end of the cytokine treatments.

Results

Four human melanoma lines of different origin and behavior were used in this study (*Table 1*). HT168-M1 and M24met are both highly metastatic variants of melanoma lines originally deriving from metastases.[27,29] WM983B, also metastasis derived, was proved tumorigenic in nude mice[31] and moderately metastatic in the spleen-liver system in SCID mice (*Table 1*). The WM35 line was derived from an early stage primary melanoma and was characterized with low tumorigenic potential.[6,31]

Using a colorimetric assay, we studied the effect of a panel of cytokines on the in vitro proliferation of the melanoma cells (*Figure 1*). TGF-β and TNF-α caused dose dependent proliferation inhibition on three of the four lines in a dose range of 0.1-10 ng/ml. IL-1α inhibited cell proliferation only in the highest dose used (10 ng/ml). M24met cells showed a decreased sensitivity to all these cytokines. The three other cytokines studied, IL-2, IL-10 and IL-12 were ineffective or slightly inhibitory at the highest dose (*Figure 1*).

The effect of cytokines on type IV collagenase production was also cell line dependent (*Figure 2*). In HT168-M1 and M24met cells, TGF-β and to a lesser extent, IL-1α caused an increased MMP-2 production, while TNF-α enhanced MMP-9 activity. No gelatinase secretion could be induced in WM35 cells by TGF-β, however, MMP-9 production was detected after TNF-α treatment, and a weaker reaction after IL-1α treatment. A different pattern of gelatinolytic activity was observed in WM983B cells. These cells reacted to TNF-α and IL-1α with a slight increase in MMP-9 production, while TGF-β stimulated the appearance of a pronounced 92 kDa band of clearing (MMP-9), a weaker 72 kDa band (MMP-2), and also higher molecular weight gelatinolytic activities. The effect of TGF-β, TNF-α and IL-1α on gelatinase production by these melanoma cell lines was dose dependent; two examples are shown on *Figure 3*. IL-2, IL-10 and IL-12 did not change MMP activities in the melanoma cell lines.

Table 2 summarizes the effects of these cytokines on the proliferation and type IV collagenase production of the melanoma cells.

Discussion

Cytokines, secreted either by the tumor cells or by host cells, have been demonstrated to play an important role as mediators and regulators of tumor – host interactions leading to the progression of malignant tumors. Many cytokines can directly influence tumor cell proliferation. Some of these cytokines, TGF-β, TNF-α, IL-1α and IL-6, have been reported to function as negative growth regulators on melanocytes and melanoma cells. However, cell lines isolated from advanced stage lesions, or tumorigenic variants of early stage melanomas, appeared to possess an enhanced resistance to these factors ("multicytokine resistance").[4,6]

In our experiments, of the three melanoma lines deriving from metastatic tumors only one, M24met showed a comparatively high resistance to all three antiproliferative cytokines studied (TGF-β, TNF-α, IL-1α). On the other hand, HT168-M1, a cell line selected for high metastatic capacity in immunosuppressed mice and characterized with

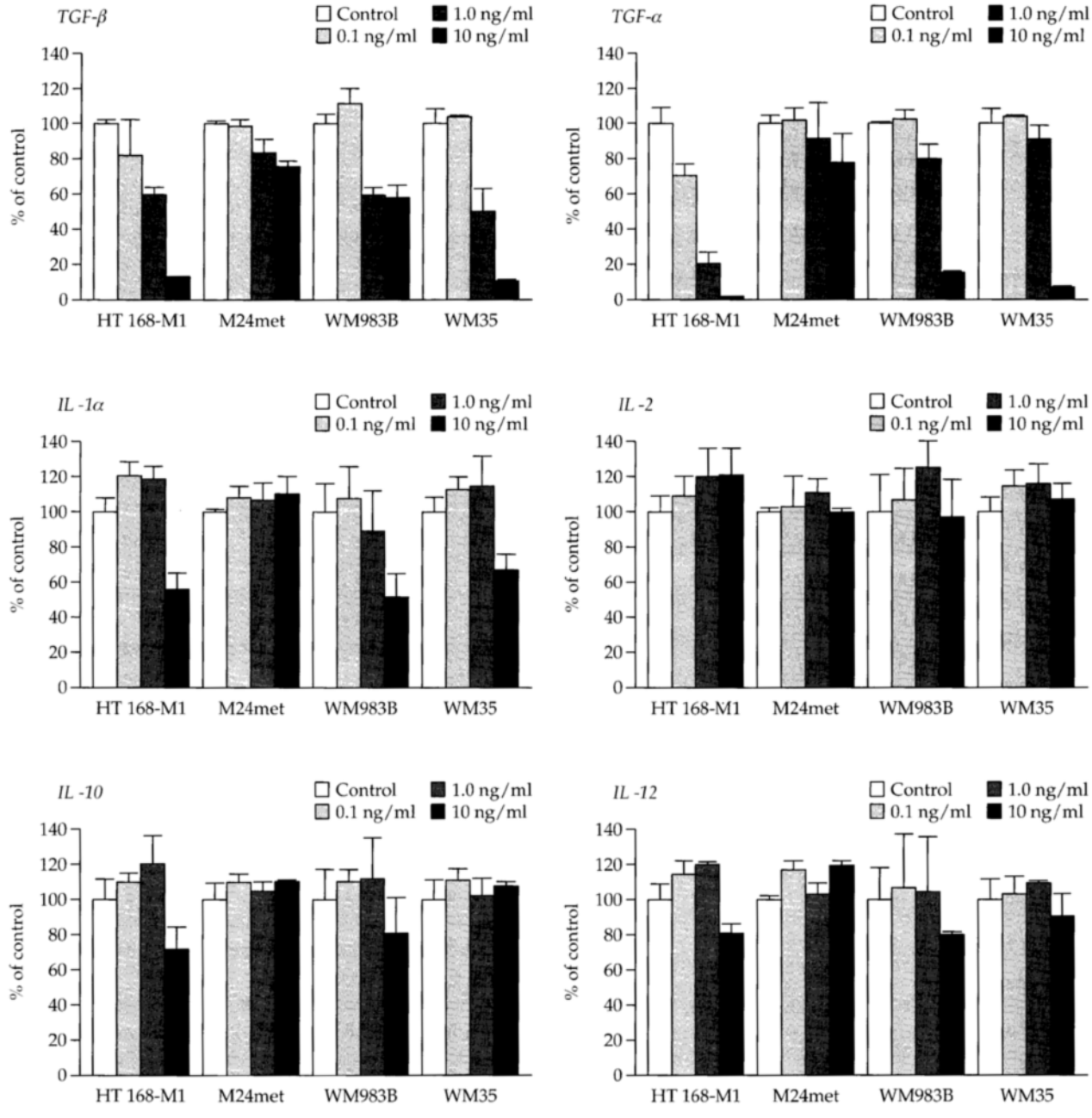

Figure 1. *Effect of cytokines on the proliferation of human melanoma cells, determined after 4 days by the MTT test. Data are given as % of control (mean±SD of 3-5 parallel samples; results of a representative experiment of two to five separate experiments).*

a very aggressive biological behavior, was one of the most sensitive lines to all three cytokines. Several other cell lines from advanced stage primary or metastatic melanomas, tumorigenic and metastatic in SCID mice, repeatedly demonstrated similar sensitivity to these cytokines (data not shown), indicating that cytokine resistance of advanced stage melanoma cells is relative and cannot be generally applied for every cell line and tumor in this category.

Two other cytokines used in our experiments, IL-2 and IL-12 are both potent stimulators of T- and NK-cell functions. These cytokines have been reported to have antitumoral effects on several murine tumors,[34,35] and are applied in clinical trials as well. Their effects are thought to be exerted principally via stimulating the immune system of the host, although there are occasional reports on direct effects on tumor cells, including melanomas.[36,37] In this study these cytokines had only marginal effect on the proliferation or gelatinase production of the melanoma cell lines.

The role of IL-10 in the tumor-bearing organism is more controversial. It was described as a cytokine

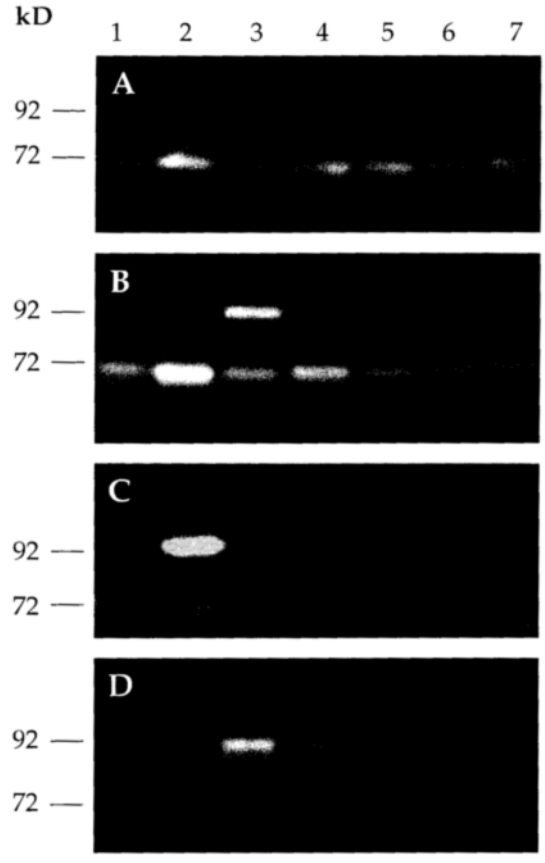

Figure 2. *Zymographic analysis of conditioned medium from HT168-M1 (A), M24met (B), WM983B (C) and WM35 (D) melanoma cell lines treated with different cytokines for 48 h in serum-free medium. 1: control, 2: TGF-β (5 ng/ml), 3: TNF-α (10 ng/ml) 4: IL-1α (10 ng/ml), 5: IL-2 (100 U/ml), 6: IL-10 (10 ng/ml), 7: IL-12 (10 ng/ml)*

inhibiting several different cell functions of the immune system, including T-, NK-cell and monocyte/macrophage functions. However, it also acts as a co-stimulator of T-cell proliferation, and maintains B-cell viability.[38] It was shown to exhibit antitumoral and antimetastatic activity in some experimental systems,[14,39] while predominantly negative effects have been described on *in vitro* antitumoral responses in humans.[40] Tumor cells, including melanomas have been demonstrated to produce this cytokine, and it was suggested that this could contribute to immune suppression by melanomas.[41] This negative immune modulation is thought to be principally exerted through inhibition of host cells, although IL-10 pretreatment of human melanoma cells caused downregulation of HLA class I expression and protected against T-cell mediated lysis.[42] There is no indication of an autocrine growth modulating effect of IL-10. Our experiments showed only a slight growth inhibition at the highest concentration used.

Type IV collagenase activity has been reported to contribute to tumor progression in several animal model systems.[15,16] Elevated serum or intratumoral MMP-2 levels were observed in advanced stage lung and colon carcinoma patients.[43,44] The correlation between tumor progression and MMP-2 production is not clear in melanoma, since the overexpression of this enzyme occurs in early stage of tumor progression, although the extent of it seemed to correlate with later haematogenous metastases.[45]

The effect of cytokines on type IV collagenase production by melanoma cells has not been extensively studied. The mechanisms of the stimulation of MMP activity by TGF-β, TNF-α and IL-1 were studied on one cell line, A2058.[19,20,23] MacDougall et al compared the effect of two cytokines, TGF-β and IL-1β on a panel of melanoma cell lines deriving from early and advanced stages, and concluded that only advanced stage lines showed constitutive or cytokine-inducible MMP-9 secretion.[22] They found that early stage melanoma lines, including WM35, were uninducible by these cytokines to produce MMP-9. In our experiments, however, this cell line was responsive to TNF-α, and to a lesser extent, IL-1α. This indicates that at least some early stage primary melanoma cell lines can

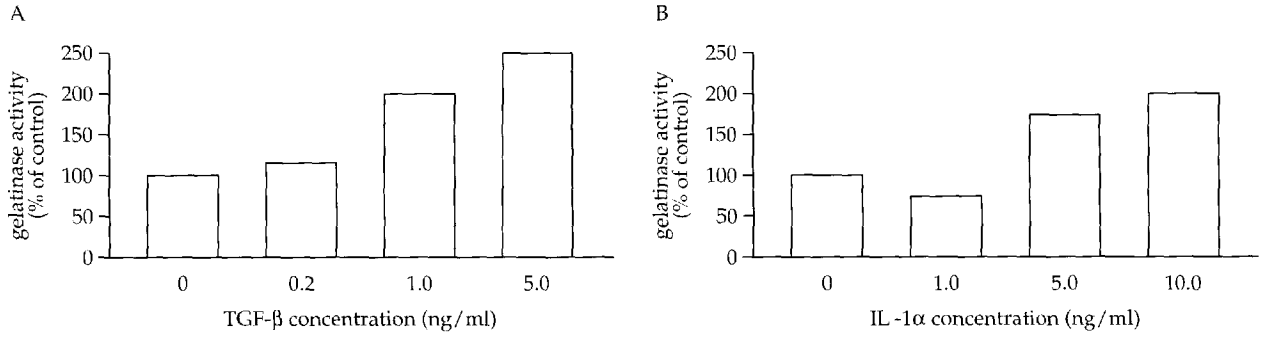

Figure 3. *Modulation of MMP-2 activity of HT168-M1 cells by TGF-β (A) or IL-1α (B). Gelatinase activities are given as % of the controls, calculated from values obtained from densitometric analysis of zymograms, corrected with cell density values deriving from SRB test.*

Table 2. **Summary of the effects of cytokines on the proliferation and gelatinase production of human melanoma cells**

	Cytokines	*Control*	*TGF-β*	*TNF-α*	*IL-1α*	*IL-2*	*IL-10*	*IL-12*
Cell lines								
HT 168-M1	proliferation		↓↓	↓↓	↓	0	(↓)	(↓)
	MMP-2	+	↑↑	0	↑	0	0	0
	MMP-9	–	0	↑↑	0	0	0	0
M24met	proliferation		(↓)	(↓)	0	0	0	0
	MMP-2	+	↑↑↑	0	↑↑	0	0	0
	MMP-9	–	0	↑↑↑	0	0	0	0
WM983B	proliferation		↓	↓	↓	0	(↓)	(↓)
	MMP-2	–	↑	0	0	0	0	0
	MMP-9	(+)	↑↑↑	↑	↑	0	0	0
WM35	proliferation		↓↓	↓	↓	0	0	0
	MMP-2	–	0	0	0	0	0	0
	MMP-9	–	0	↑↑↑	↑	0	0	0

+: detectable; –: undetectable; ↑: stimulation; ↓: inhibition; 0: no effect
cytokine concentrations are shown in *Figure 1* and *Figure 2*.

be induced to produce MMP-9, although the significance of this effect in tumor progression is not clear, since WM35 displayed little tumorigenicity in nude[6,31] or SCID mice (*Table 1*).

The results of this and other studies using different cell lines suggest that the effect of cytokines on the production of the metalloproteinase subtypes is highly cell line dependent. TNF-α and IL-1 have been demonstrated to be stimulatory mainly on the 92 kDa collagenase, MMP-9, but their effectivity showed differences depending on the cell lines.[19,20,22] In accordance with this, although TNF-α stimulated MMP-9 activity on all melanoma lines used in this study, the degree of this effect was different. IL-1α induced MMP-9 production in two of the four lines (WM983B and WM35), while in the other two lines it stimulated MMP-2 activity.

The effect of TGF-β, which has been shown to influence both MMP-2 and MMP-9,[22-24] was also different in the four cell lines.

Interestingly, the cytokines that proved antiproliferative in our experiments, also demonstrated stimulatory effect on type IV collagenase secretion. This indicates that negative and positive cytokine effects can coexist even under *in vitro* conditions. Melanomas have been reported to produce most of these factors,[46] and these tumor-derived cytokines can influence both the tumor cells and host cells through autocrine and paracrine mechanisms, contributing to the modulation of tumor progression.

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