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IMMUNOHISTOCHEMICAL EVIDENCE OF CYTOKINE NETWORKS DURING PROGRESSION OF HUMAN MELANOCYTIC LESIONS

Silvia MORETTI[1]*, Cinzia PINZI[2], Adelina SPALLANZANI[2], Emilio BERTI[3], Alessandra CHIARUGI[2], Sandra MAZZOLI[4], Massimo FABIANI[5], Carlo VALLECCHI[2] and MEENHARD HERLYN[6]

[1]*Second Dermatology Unit, S. Maria Nuova Hospital, Azienda Sanitaria di Firenze, Florence, Italy*
[2]*Institute of Dermatology Clinic, University of Florence, Florence, Italy*
[3]*I Dermatology Clinic, University of Milan, Milan, Italy*
[4]*STD Unit, S. Maria Annunziata Hospital, Azienda Sanitaria di Firenze, Florence, Italy*
[5]*Epidemiology and Biostatistic Unit, Istituto Superiore di Sanità, Rome, Italy*
[6]*The Wistar Institute, Philadelphia, PA, USA*

Melanoma cells in culture express a variety of growth factors and cytokines and some of their autocrine and paracrine roles have been investigated. However, less information is available on the potential dynamic changes in expression of these molecules on cells during melanoma development and progression *in situ*. Using immunohistochemistry, we tested 40 nevi and primary and metastatic melanoma lesions for the expression of 10 growth factors and cytokines and the respective receptors representing 10 cell surface molecules. Nevi and thin (< 1 mm) primary melanomas showed little expression of ligands except weak reactivity of tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), interleukin-8 (IL-8) and reactivity of TGF-βR and c-kit. Marked up-regulation of growth factors, cytokines and receptor expression was observed in thick (> 1 mm) primary melanomas, which were stained with polyclonal or monoclonal antibodies (MAbs) for IL-1α, IL-1β, IL-6, IL-8, TNF-α, TGF-β, granulocyte-macrophage colony-stimulating factor (GM-CSF) and stem cell factor (SCF), but not IL-2. Metastases showed similar expression patterns except that SCF was absent. Co-expression of ligand and receptor was observed for TGF-β, GM-CSF and IL-6, suggesting an autocrine role for these ligands. TNF-α appears to be a marker of benign lesions; IL-6 and IL-8 expression is associated with biologically early malignancy; TGF-β, GM-CSF and IL-1α are highly expressed in biologically late lesions; and TNF-β is an apparent marker of metastatic dissemination. Our results indicate that melanoma cells utilize cascades of growth factors and cytokines for their progression. *Int. J. Cancer* (*Pred. Oncol.*) 84:160–168, 1999.

Human tumor cells constitutively produce a variety of cytokines and growth factors. During tumor progression, neoplastic cells continuously decrease their need for exogenous stimulating factors, until they achieve complete independence from these mitogens. Melanoma cells in culture express a large number of cytokines and growth factors (Mattei *et al*., 1994; Rodeck *et al*., 1991). Ligand production and receptor expression are at times concomitant in melanoma cells, suggesting a possible autocrine control mechanism, and evidence suggests that some melanoma-derived cytokines and growth factors may exert paracrine effects on stromal cells and host immune and inflammatory cells. Conversely, cytokines and growth factors produced by normal cells in tumor stroma can stimulate malignant cells (Herlyn, 1993). Thus, cytokines and growth factors in the cutaneous melanoma microenvironment may regulate functions of both malignant and stromal cells, possibly affecting the mechanism of tumor progression. Such crosstalk requires the expression of suitable receptors on either the malignant or normal cells. However, most studies on autocrine and paracrine growth factors have been done with cultured cells and little is known about potential regulatory pathways in melanocytic lesions *in situ*.

Melanoma progression appears to be closely associated with increasing growth autonomy of the malignant cells, apparently due to the constitutive production of growth factors and their respective receptors. RNA transcripts for transforming growth factor (TGF)-β and Steel factor or stem cell factor (SCF) have been demonstrated in melanoma cells, nevus cells and melanocytes, whereas interleukin (IL)-1α, IL-1β, IL-6, IL-8, gro-α, Rantes, interferon (IFN)-β, tumor necrosis factor (TNF)-α, TNF-β, G-colony stimulating factor (CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and platelet-derived growth factor (PDGF)-A and PDGF-B have been detected only on melanoma cells (Albino *et al*., 1991; Amstrong *et al*., 1992; Colombo *et al*., 1992, Mattei *et al*., 1994; Rodeck *et al*., 1991). Receptor (R) signals for epidermal growth factor (EGF), fibroblast growth factor (FGF), nerve growth factor (NGF) p70 and c-kit were found on melanocytes and melanoma cells; and IL-6R, TNFR, GM-CSFR, and CXCR2, the receptor for IL-8, were detected on melanoma cell lines (Mattei *et al*., 1994; Norgauer *et al*., 1996). Production of IL-1α, IL-1β, IL-2, IL-6, IL-8, TGF-β, and GM-CSF has also been observed in melanoma cell lines (Alileche *et al*., 1993; Amstrong *et al*., 1992; Bennicelli and Guerry, 1993; Colombo *et al*., 1992; Herlyn, 1993; Rodeck *et al*., 1994). *In situ*, far less information is available. RNA transcripts for TGF-β isoforms (Reed *et al*., 1994; Schmid *et al*., 1995; van Belle *et al*., 1996) or protein expression of TGF-β (Moretti *et al*., 1997; Schmid *et al*., 1995), IL-1α and IL-1β (Ahmed *et al*., 1995; Tyler *et al*., 1995), IL-2 (McMillan *et al*., 1995), IL-8 (Gutman *et al*., 1995), IL-6 and TNF-α (Ahmed *et al*., 1995) have been described. Even less is known about receptor expression, and studies are limited to proteins such as epidermal growth factor receptor (EGFR, De Wit *et al*., 1992), c-kit (Natali *et al*., 1992), IL-2R (McMillan *et al*., 1995), and TGF-β type IIIR (Moretti *et al*., 1997) or to mRNA transcript of TGF-β type IIR (Schmid *et al*., 1995).

In the present study, we analyzed the expression of growth factors, cytokines, and their respective receptors in melanocytic lesions, because (1) growth factor and cytokine expression may affect the biological evolution of progression; (2) expression of the respective receptors may give insight into the possible autocrine nature of a given ligand; (3) cytokines with known functions for inflammatory and immune cells might influence host defense mechanisms; (4) expression of growth factors with known functions for stromal fibroblasts and endothelial cells might mean that stimulation of the stroma is important at a given stage; and (5) testing multiple growth factors, cytokines and their receptors for expression might provide information on potential common pathways suggestive of functional networks and cross-talk. Thus, information on expression should provide guidance for in-depth experimental functional analyses. We have found an overall an

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*Correspondence to: Second Dermatology Unit, S. Maria Nuova Hospital, Azienda sanitaria di Firenze, via della Pergola 58, 50121, Florence, Italy. Fax: (39)55–2347374.

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TABLE I – CLINICAL AND HISTOLOGICAL INFORMATION ON MELANOCYTIC LESIONS

Lesion	Number	Parameter	Number	Histologic type[1]	Number
Nevus	10	Average age	24 years	Dermal nevus	2
		Males	7	Congenital nevus	2
		Females	3	Compound nevus	3
				Dysplastic nevus	2
				Spitz nevus	1
Primary melanoma[2]	20	Average age	60 years	Superficial spreading	16
		Males	8	Lentigo malignant	1
		Females	12	Acral lentiginous	1
				Unclassified	1
				Nodular	1
				Clark level	
				I	1
				II	3
				III	10
				IV	5
				V	1
				Thickness (mm)	
				<1.0	7
				>1.0	13
Metastatic melanoma	10	Average age	65 years	Subcutaneous/skin	5
		Males	4	Lymph node	5
		Females	6		

[1]Established according to Clark *et al.* (1986).–[2]All primary melanomas were stage I, according to the ITNM classification (UICC, 1987).

TABLE II – ANTIBODY PANEL

Reactivity	Clone/specificity	Working dilution	Source/donor
Monoclonal antibodies			
Type 1 hIL-1R	hIL1R-M1	1:100	Armitage[1]
Type 2 hIL-1R	hIL1R2-M22	1:100	Armitage[1]
55 kDa IL-2R	Anti-TAC	1:20	Miyasaka[1]
IL-6Rα	B-H23	1:100	Wijdenes[1]
IL-8Rα	B-F25	1:30	Wijdenes[1]
SCFR	17F11	1:200	Van Agthoven[1]
GM-CSFRα	hGMCSFR-M11	1:1	Armitage[1]
55 kDa TNFR	MR1-3	1:200	Buurman[1]
75 kDa TNFR	MR2-1	1:200	Buurman[1]
Type III TGF-βR	1G2	1:200	Buhring[1]
gp130-IL-6 signal	AM64	1:100	Kishimoto[1]
SCF mb[2]	4B10	1:100	Pietsch[1]
IL-8	Anti-IL-8	1:20	Genzyme
GM-CSF	DF2714	1:100	Flavell[1]
TNF-α	Anti-TNF-α	1:100	Genzyme, Cambridge, MA
TGF-β1-2-3	Anti-TGF-β1-2-3	1:200	Genzyme
IL-6	Anti-IL-6	1:50	Genzyme
IL-1α	Anti-IL-1α	1:20	Oncogene Science, Manhasset, NY
IL-1β	Anti-IL-1β	1:20	Oncogene Science
Polyclonal antibodies			
TNF-β	Anti-TNF-β	1:200	Genzyme
IL-2	Anti-IL-2	1:100	Genzyme

R, receptor; IL, interleukin; SCF, stem cell factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; TNF, tumor necrosis factor; TGF-β, transforming growth factor β.–[1]See Letarte *et al.* (1995).–[2]mb = membrane-bound.

increase in expression with progression, suggesting an increased utilization of growth factors and cytokines for self stimulation, and occasional discordant expression of ligands and receptors, suggesting the existence of paracrine regulatory pathways between malignant cells and normal cells in the tumor microenvironment.

MATERIAL AND METHODS

Tissue samples

Fresh specimens were obtained from 10 melanocytic nevi (3 compound, 2 intradermal, 2 congenital, 2 dysplastic nevi and 1 Spitz nevus), 20 primary cutaneous melanomas and 10 metastatic melanomas; primary melanomas were separated into 2 groups according to thickness: < 1 mm ($n = 7$) and > 1 mm ($n = 13$). Table I gives clinical and pathological information on these patients.

Immunohistochemistry

Immunostaining was carried out using an alkaline phosphatase-anti-alkaline phosphatase (APAAP) method whereas murine antibodies served as primary reagents; most primary antibodies in our study were mouse monoclonal antibodies (MAbs). Table II lists the source, reactivity and working dilution of the antibodies used. Sections were incubated with human AB-positive serum followed by primary mouse MAb for 1 hr at room temperature, rabbit anti-mouse (RAM) IgG (Dako, Glostrup, Denmark), and preformed murine monoclonal APAAP soluble complexes (Dako). The RAM/APAAP incubations were repeated 3 times, because the

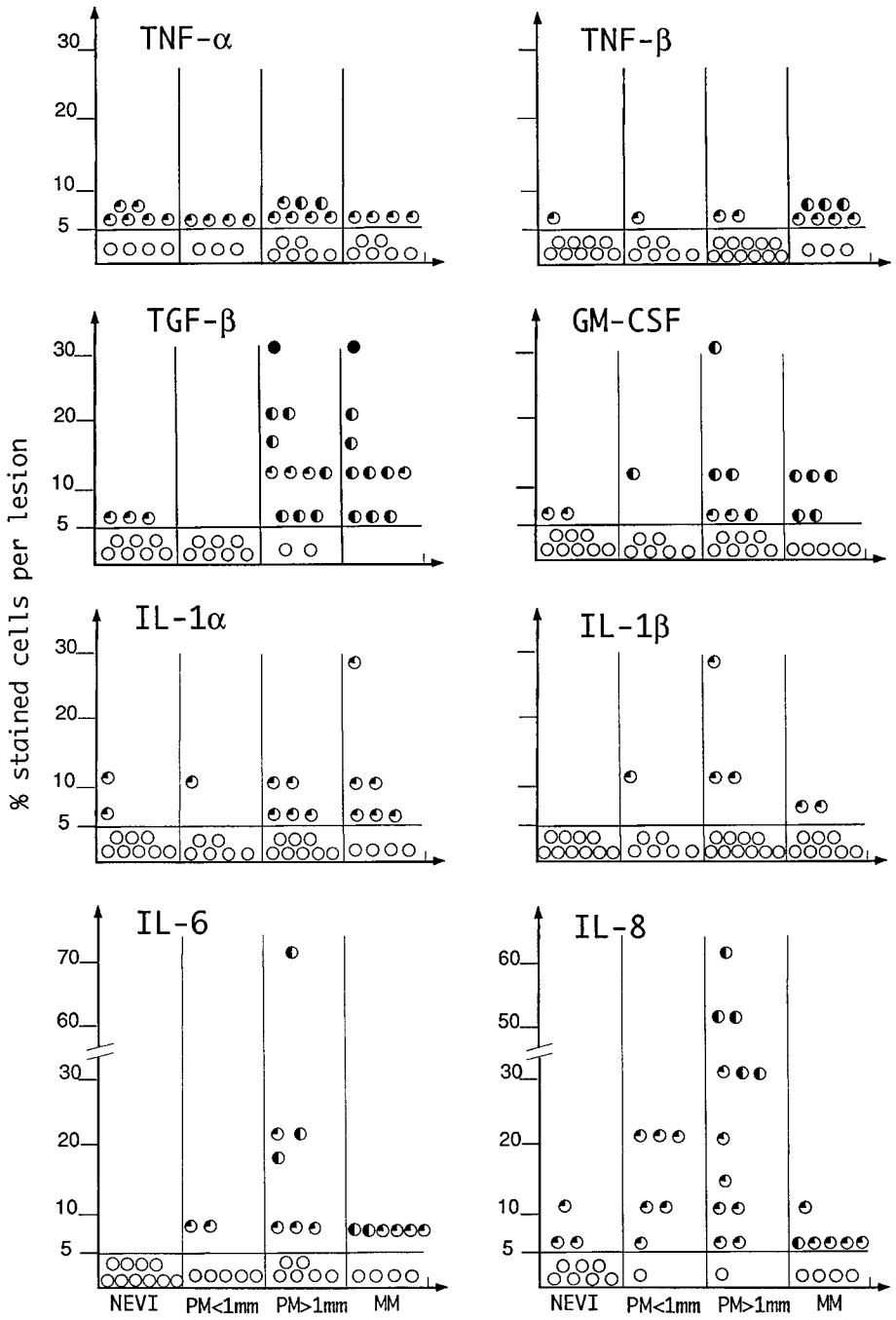

FIGURE 1 – Expression of cytokines on nevi, primary melanomas (PM) less than 1 mm thick or thicker than 1 mm, and metastatic melanomas (MM). Each circle represents the percentage of stained cells for one lesion. An open circle indicates negative staining; a quarter-black circle indicates positive staining at + intensity; half-black circle indicates positive staining with ++ intensity; a solid-black circle indicates staining with +++ intensity.

expression of cytokines, growth factors and receptors was expected to be low in tissues. Alkaline-phosphatase substrate (hexazotized new fuchsin and AS-B1-sodium salt; Sigma, St. Louis, MO) was then applied to the sections. Control sections in each experiment were incubated with normal mouse IgG. Positive controls for each cytokine were: staining of macrophages as internal control for IL-1α and IL-1β, TNF-α, TGF-β, GM-CSF, IL-6, and IL-8; staining of mast cells in the dermis of eczematous skin as positive control for SCF. Positive controls for receptor staining were: staining of macrophages (for IL-1R-type 1 and 2, 75 kDa/ and 55 kDa/TNFR, CXCR-1, GM-CSFRα, IL-6Rα), fibroblasts (for IL-6Rα), blood vessels (for TGFβR type III), lymphocytic infiltrate (for 55 kDa/IL-2R), or epidermal melanocytes (for SCFR or c-kit) as internal controls. MAb against gp130 was also tested on lesions reacting with anti-IL-6R antibodies.

When primary polyclonal antibodies were of rabbit origin (anti-TNF-β and anti-IL-2), a streptavidin-biotin alkaline-phosphatase staining method was used. Sections were incubated with normal swine serum, primary reagent, a biotinylated swine anti-rabbit IgG antibody, and finally with streptavidin-conjugated alkaline-phosphatase (all from Dako). The reaction was developed as described above. Control sections were incubated with normal rabbit IgG. The specificity of polyclonal anti-TNF-β antibody was confirmed by pre-incubation with recombinant human (rh)TNF-β at 4°C followed by centrifugation (10,000 *g* for 5 min) before use.

TABLE III – STATISTICAL COMPARISON OF EXPRESSION OF GROWTH FACTORS AND CYTOKINES IN MELANOCYTIC LESIONS

	(E + A + M) *vs.* N[1]			(A + M) *vs.* E			M *vs.* (E + A)			M *vs.* N		
	OR[2]	C.I.[2]	*p*	OR	C.I.	*p*	OR	C.I.	*p*	OR	C.I.	*p*
TNF-α	0.7	0.1–3.5	NS[3]	0.7	0.1–5.2	NS	0.5	0.1–3.2	NS	0.4	0.1–3.6	NS
TNF-β	4.5	0.5–216.6	NS	3.9	0.4–196.8	NS	13.2	1.6–120.4	0.005	21.0	1.4–1047.8	0.020
TGF-β	5.4	0.9–38.4	NS	NE	NE	<0.001	NE	NE	0.013	NE	NE	0.003
GM-CSF	2.8	0.4–31.2	NS	6.0	0.5–300.6	NS	1.7	0.3–10.5	NS	4.0	0.4–53.8	NS
IL-1α	2.7	0.4–29.4	NS	5.5	0.5–275.5	NS	3.5	0.6–23.1	NS	6.0	0.6–80.0	NS
IL-1β	NE[4]	NE	NS	1.7	0.1–91.8	NS	1.0	0.1–8.9	NS	NE	NE	NS
IL-6	NE	NE	0.007	2.7	0.3–33.2	NS	1.2	0.2–7.3	NS	NE	NE	0.033
IL-8	9.3	1.5–68.5	0.006	0.6	0.0–7.3	NS	0.2	0.0–1.6	NS	3.5	0.4–33.3	NS

TNF, tumor necrosis factor; TGF, transforming growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin.–[1]N, nevi; E, early primary melanoma; A, advanced primary melanoma; M, metastasis.–[2]OR, odds ratio, calculated based on a 2-by-2 Table describing how the different groups of 2 dichotomous variables are associated. C.I., confidence intervals.–[3]NS, not significant.–[4]NE, not estimable (if expression in at least one group was not detected).

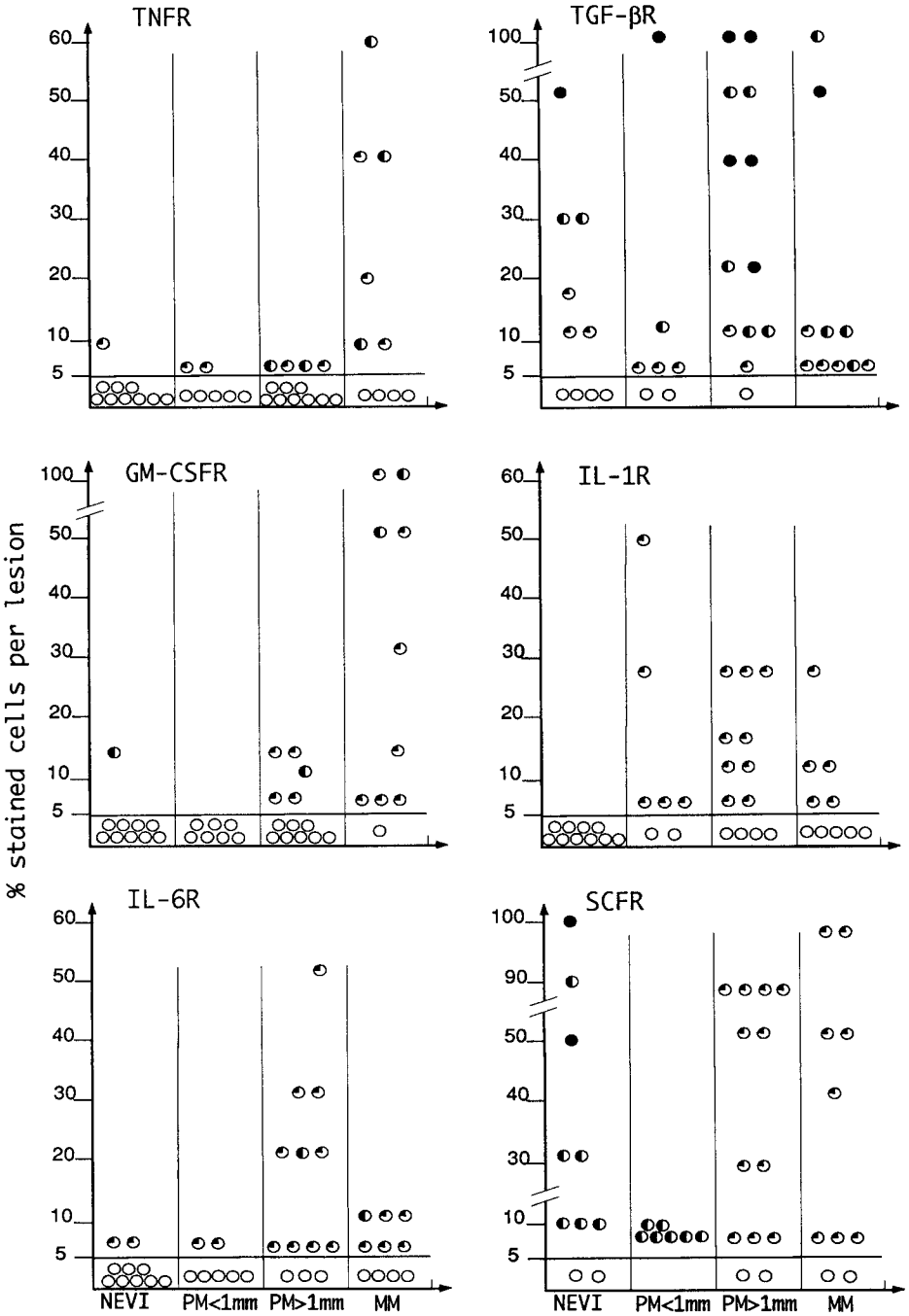

FIGURE 2 – Expression of cytokine receptors on nevi, primary melanomas (PM) less than 1 mm thick or thicker than 1 mm, and metastatic melanomas (MM). Symbols same as in Figure 1.

TABLE IV – STATISTICAL COMPARISON OF EXPRESSION OF RECEPTORS FOR GROWTH FACTORS AND CYTOKINES IN MELANOCYTIC LESIONS

	(E + A + M) *vs.* N[1]			(A + M) *vs.* E			M *vs.* (E + A)			M *vs.* N		
	OR[2]	C.I.[2]	*p*	OR	C.I.	*p*	OR	C.I.	*p*	OR	C.I.	*p*
TNFR	6.0	0.7–284.5	NS[3]	1.9	0.2–23.7	NS	3.5	0.6–23.1	NS	13.5	1.0–687.9	NS
TGF-βR	6.0	0.8–49.8	NS	8.8	0.4–541.9	NS	NE	NE	NS	NE	NE	NS
GM-CSFR	7.9	0.9–369.3	NS	NE	NE	0.007	27.0	2.4–1259.2	0.001	81.0	3.2–3920.2	0.001
IL-1R	NE[4]	NE	0.001	0.6	0.1–5.0	NS	0.4	0.1–2.7	NS	NE	NE	0.033
IL-6R	6.0	0.9–64.6	NS	5.7	0.7–69.3	NS	1.0	0.2–6.5	NS	6.0	0.6–80.0	NS
SCFR	1.6	0.1–13.9	NS	NE	NE	NS	0.4	0.0–7.4	NS	1.0	0.1–17.1	NS

R, receptor; TNF, tumor necrosis factor; TGF, transforming growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin.–[1]N, nevi; E, early primary melanoma; A, advanced primary melanoma; M, metastasis.–[2]Odds ratio (OR) and confidence intervals (C.I.) as in Table III.–[3]NS, not significant.–[4]NE, not estimated (see Table III).

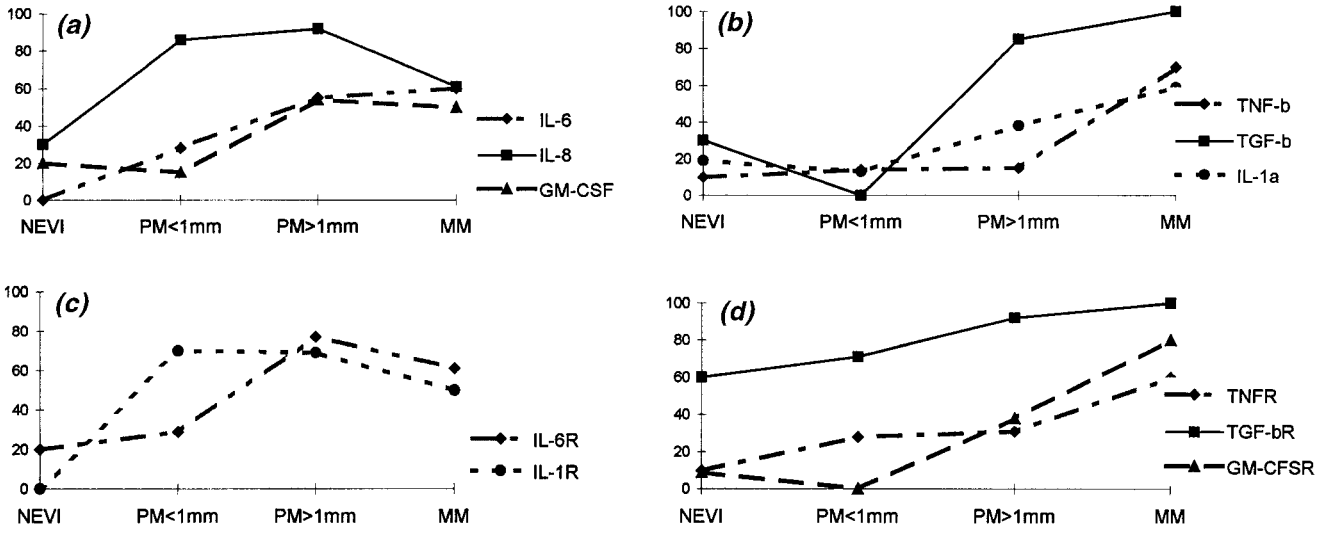

FIGURE 3 – Pattern of cytokine expression on nevi, primary melanomas (PM) less than 1 mm thick or thicker than 1 mm, and metastatic melanomas (MM). Each point represents the percentage of positive lesions. (*a*) Expression of IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF); (*b*) tumor necrosis factor-β (TNF-β), transforming growth factor-β (TGF-β), and interleukin-1α (IL-1α); (*c*) IL-6R, IL-1R; and (*d*) receptor (R) signals for TNFR, TGF-βR and GM-SCFR.

Staining of macrophages served as positive control for both TNF-β and IL-2. All sections were counterstained with hematoxylin.

Evaluation of results

Two investigators (SM and CP) read all tissue sections. The percentage of positive cells per lesion was scored according to 5 different categories: 0–4%, 5–10%, 11–30%, 31–50% and 51–100%. A cutoff of 5% stained cells was chosen to identify positive lesions. Discrepancies in the reading were resolved by a second parallel reading of the slides. At least 5 fields (×400) per section were examined. Staining intensity was graded as +/++/+++ (*i.e.*, weak/moderate/high). Staining for growth factors and cytokines was assessed as positive when melanocyte/melanoma cells showed reactivity in the cytoplasm.

The MAbs HMB45 (Enzo, Farmingdale, NY) and 225.28 (generous gift of Dr. S. Ferrone, Valhalla, NY) were used to identify melanoma and nevus cells in tissue sections; anti-CD3 and anti-CD68 (Dako) MAbs served to identify lymphocytic infiltrate and macrophages, respectively.

For statistical analyses, non-parametric tests were used to determine significant differences between groups. The distribution of the scored percentages of positive lesions after immunohistological staining in each group was the unit of analysis. The proportion of positive lesions was compared between different subgroups of the study population for each cytokine and receptor. Subgroups included the 4 different groups of lesions: nevi, primary melanomas $<$ 1 mm thickness, primary melanomas $>$ 1 mm thickness and metastases. Statistical evaluation was performed comparing: (a) benign lesions (nevi) *vs.* malignant (primary and metastatic) lesions; (b) thin primary melanomas (early lesions) *vs.* thick primary and metastatic melanomas (advanced lesions); (c) primary *vs.* metastatic melanomas; and (d) nevi *vs.* metastatic melanomas. Statistical significance of the differences in proportions was evaluated using the Yates corrected chi-square or the Fisher exact test when appropriate. Differences were considered significant at $p < 0.05$.

RESULTS

Growth factor and cytokine expression

We tested 40 different lesions representing 4 stages of tumor progression for reactivity with antibodies recognizing 10 different cytokines and growth factors. Figure 1 shows the percentage of cells reactive with each lesion and the relative staining intensity. Table III compares reactivity between the different groups of lesions. TNF-α expression was detected in a low percentage of cells in the majority of nevi and thin melanomas and in nearly half of the thick primary and metastatic melanomas (Fig. 1); no statistical difference between any of the 4 groups of lesions was evident (Table III). Expression of 7 cytokines (TNF-β, TGF-β, GM-CSF, IL-1α, IL-1β, IL-6, IL-8) increased with tumor progression. TNF-β was detected in a low percentage of cells per lesion, and was expressed weakly in nevi and primary melanomas, but expressed highly in metastases (nevi *vs.* metastases, $p = 0.02$; primary melanomas *vs.* metastases, $p = 0.005$). TGF-β was found in 3 nevi, but not on thin primary melanomas, whereas 11 of 13 thick primary melanomas and all 10 metastases expressed TGF-β (early *vs.* advanced lesions, $p = 0.00001$; primary *vs.* metastatic

melanomas, $p = 0.013$; nevi *vs*. metastases, $p = 0.003$). GM-CSF and IL-1α were detected in a minority of nevi and thin primary melanomas, and on nearly half of the thick (in this case, > 1.5 mm) primary melanomas and metastases, without significant differences. A low to medium percentage of cells per lesion reacted with MAbs to these 2 cytokines. IL-1β was expressed in only a few primary melanomas and metastases, and expression was variable. IL-6 was absent in all nevi but increased from thin primary to metastatic melanomas, and a low to medium percentage of cells was stained (benign *vs.* malignant lesions, $p = 0.007$; nevi *vs.* metastases, $p = 0.033$). Expression of IL-8 in nevi was increased in thin and thick primary melanomas. In metastases, the percentage of stained cells decreased sharply (benign *vs*. malignant lesions, $p = 0.006$), and low, medium and high percentages of stained cells were seen.

Staining intensity was assessed as weak (+) or moderate (++), whereas high intensity (+++) was rare. Highest staining intensities were seen for TGF-β, GM-CSF, IL-8 and IL-6. IL-2 showed no reactivity on melanocytic cells (whereas macrophage staining was commonly observed), and SCF was expressed weakly in nevi (2 out of 10) and in primary melanomas (1 thin and 2 thick lesions) and was not expressed in metastases (not shown).

Cytokine receptor expression

The expression of 5 receptors (TNFR, TGF-βR type III, GM-CSFR, IL-1R types 1 and 2, and IL-6R) increased with tumor progression (Fig. 2). The 2 types of TNFRs, 55 and 75 kDa, were similarly expressed on a small number of lesions, with increases in advanced primary melanomas and metastases (Fig. 2). Among nevi, only one Spitz nevus showed reactivity. TGF-β type IIIR was detected in all stages of melanocytic lesions, and expression increased with tumor progression; most positive lesions showed a medium to high percentage of stained cells. GM-CSFR was not detected on nevi (only the Spitz nevus exhibited reactivity) or thin primary melanomas, but was increasingly expressed in thick melanomas and metastases (early *vs*. advanced malignant lesions, $p = 0.007$; primary *vs*. metastatic melanomas, $p = 0.001$; nevi *vs*. metastases, $p = 0.001$) (Table IV). IL-1 type 1 and type 2 receptors were similarly expressed, staining a low to medium percentage of cells, and were detected in the majority of malignant lesions, but were not detected in nevi (benign *vs*. malignant lesions, $p = 0.001$; nevi *vs*. metastases, $p = 0.033$). IL-6R was expressed weakly in 2 nevi and 2 thin primary lesions and increased, though not significantly, in (> 1.5 mm) primary and metastatic melanomas, where a low to medium percentage of cells per lesion showed staining. Interestingly, IL-6R-positive lesions were also positive for gp130, which often exhibited a nuclear staining pattern. SCFR reactivity decreased with tumor progression, with highest expression detected in nevi (the only negative lesions were 2 dermal nevi), whereas the percentage of stained melanocytic cells per lesion and the number of positive lesions did not differ significantly between thick primary and metastatic melanomas. IL-2R showed no reactivity on melanocytic cells, whereas staining was found on the lymphocytic infiltrate in most lesions. No reactivity of IL-8R was observed on melanocytic cells. Staining intensity was commonly assessed as low to moderate (+ to ++), and only SCFR (in nevi) and TGF-β type IIIR (in nevi and melanomas) exhibited high intensity (+++).

From the overall data, we conclude that nevi show the least growth factor receptor reactivity (Fig. 3), with the exception of SCFR, which was detected at the highest levels in nevi (Fig. 2). Of the cytokines, nevi express TGF-β, TNF-α and IL-8 in a low percentage of lesions (Fig. 3).

Co-expression of ligand and receptors

Co-expression of ligand and receptor on the same nevus was generally not found, except in one Spitz nevus for TNF-α, TGF-β, GM-CSF, SCF and in dysplastic nevi for TGF-β. Thin primary melanomas also showed little expression of cytokines and receptors; the most frequently expressed cytokines were TNF-α and IL-8, and ligands and receptors were generally not co-expressed. In thick primary melanomas, all cytokines except IL-2 and all receptors except IL-2R and IL-8R were detected. The cytokines TNF-α, IL-8, TGF-β, GM-CSF and IL-6, and the receptors TGF-βR, SCFR, IL-6R and IL-1R were the most frequently expressed in thick melanomas; co-expression of ligand and receptor was found for TGF-β and IL-6, and in some lesions, GM-CSF. In metastases, all cytokines except IL-2 and SCF, and all receptors except IL-2R and IL-8R were expressed. The cytokines TGF-β, IL-8, TNF-β, IL-6, IL-1α and GM-CSF, and the receptors TNFR, TGF-βR, GM-CSFR, IL-6R and SCFR were frequently expressed; co-expression of ligand and receptor was observed for TGF-β, GM-CSF and IL-6. Significant differences between lesions were observed for expression of the cytokines TNF-β, TGF-β, IL-8 and IL-6 (Table III), and for the receptors IL-1R and GM-CSFR (Table IV).

DISCUSSION

The immunohistochemical evaluation of 4 groups of melanocytic lesions, *i.e*., nevi, thin (< 1 mm) and thick (> 1 mm) primary melanomas, and metastatic melanomas, revealed distinct expression patterns for cytokines and their receptors that have potential significant biological consequences for tumor progression and disease outcome. Three overall patterns of expression were noted for the 10 cytokines and 8 receptor types representing 10 cell surface molecules: (1) increased expression with tumor progression by 8 of 10 ligands and 5 of 8 receptors; (2) decreased expression with progression, which was seen with only one molecule (SCFR as c-kit); and (3) no reactivity, which was observed only for IL-2 and its receptor and IL-8R. Between ligand and receptor expression, we observed 3 patterns in melanoma cells: (1) concomitant expression of both; (2) expression of ligand only; and (3) expression of receptor only. Some of these molecules may serve as progression markers to distinguish malignant from non-malignant lesions, and may be useful in predicting disease outcome. For example, IL-6 and IL-8 appear to be markers of malignant transformation and to be more frequent on melanomas; TGF-β is a marker of advanced progression, based on its high level of expression in both thick primary and metastatic melanomas; and TNF-β is a marker of metastases. Among receptors, IL-1R appears to be a marker of malignancy, because it is highly expressed in melanomas, and GM-CSFR is a marker of late progression, because it is found mainly in thick primary and metastatic melanomas.

Our results are consistent with previous ones on melanocytic lesions indicating decreased expression of SCFR (Natali *et al*., 1992), increased TGF-βR type III (Moretti *et al*., 1997) and increased mRNA levels of IL-1α, IL-1β, IL-6, IL-8 and GM-CSF (Ciotti *et al*., 1995) with progression. Whereas melanoma, like many tumors, often expresses growth factors and cytokines without the need for activation by exogenous ligand, it is likely that several melanoma-produced cytokines are activated by other endogenous ligands or by ligands released from juxtaposed normal host cells. For example, IL-1 and TNF-α can induce the expression of both IL-8 and IL-6 (Colombo *et al*., 1992). However, the focal expression of TNF-α, TNF-β, IL-1α and IL-1β in some lesions would not suggest the up-regulation of other cytokines expressed over the entire lesion. Double staining for 2 or more cytokines in the same sections might answer this question. The relative heterogeneity of melanomas with respect to cytokine expression, except for TGF-β and IL-8, suggests that none of the cytokines are essential for survival and growth. However, each cytokine is expected to have defined functions and perhaps also overlapping functions with other cytokines (Mattei *et al*., 1994).

The concomitant expression of ligand and receptor implies that these cytokines have an autocrine function(s), which could be related to growth or different steps of invasion, including motility, proteolysis and adhesion. An autocrine growth stimulatory role has been suggested for IL-8 (Schadendorf *et al*., 1993). IL-6 can stimulate melanoma cells from advanced but not biologically early primary melanomas (Lu and Kerbel, 1993). A potential autocrine

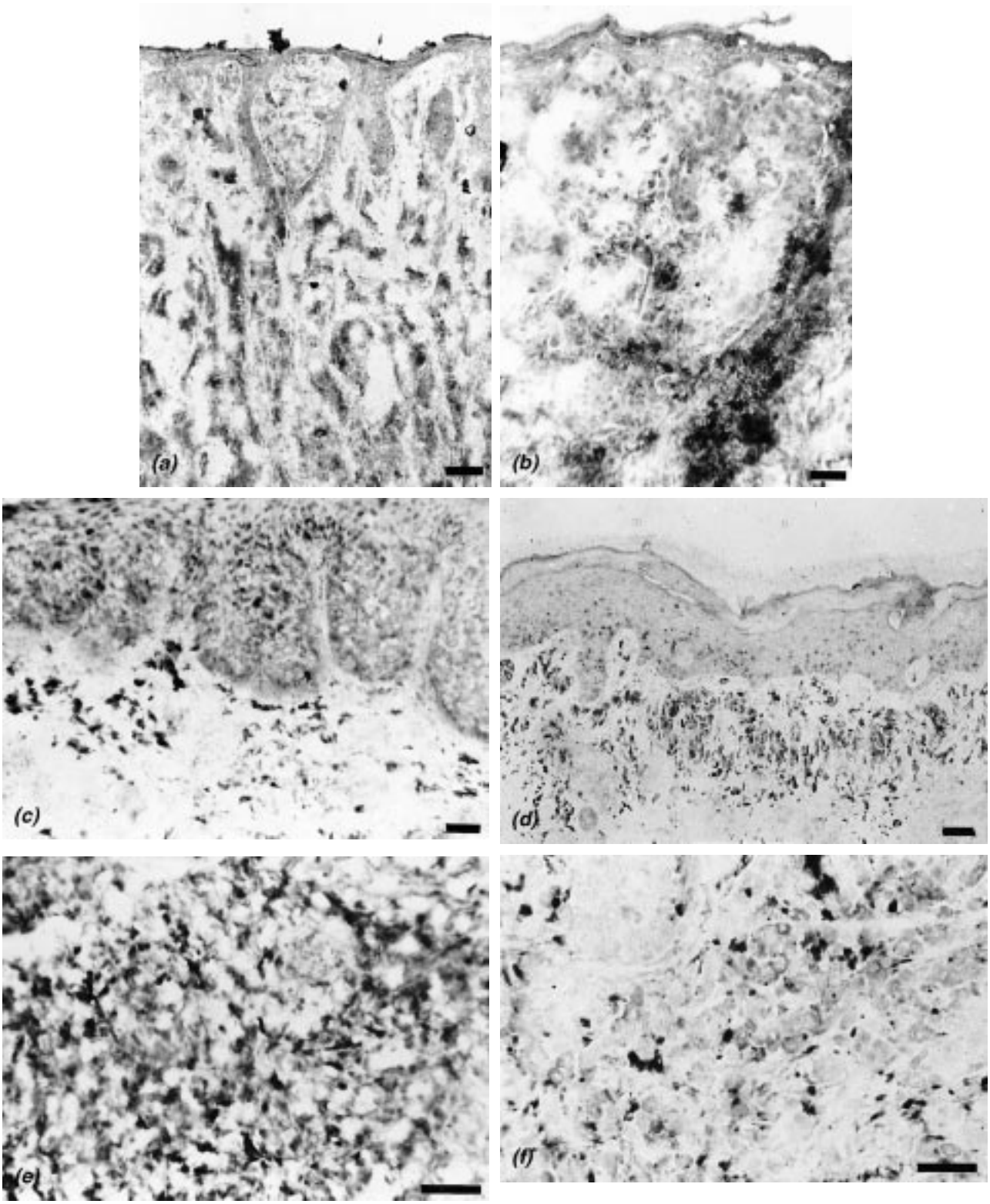

FIGURE 4 – Cytokine and cytokine receptor staining of human melanocytic lesions. Tumor necrosis factor-α (TNF-α) staining of a congenital nevus (*a*); interleukin (IL)-8 reactivity of a thick (*b*) and thin (*c*) primary melanoma; IL-6 and IL-6R staining of a thin primary melanoma (*d* and *g*, respectively) and of a metastasis (*e* and *h*, respectively); reactivity of granulocyte-macrophage colony-stimulating factor (GM-CSF) (*f*) and the receptor GM-CSFR (*i*) on a metastatic lesion. Scale bar: 20 μm.

function for IL-6 is suggested by the fact that thick (late) but not thin (early) primary melanomas express the receptor. The role of TGF-β as a potential autocrine growth factor is more complex and we cannot draw conclusions from our results because TGF-β is generally secreted in an inactive form and requires prior activation (Rodeck *et al*., 1994). The TGF-β type III receptor evaluated here

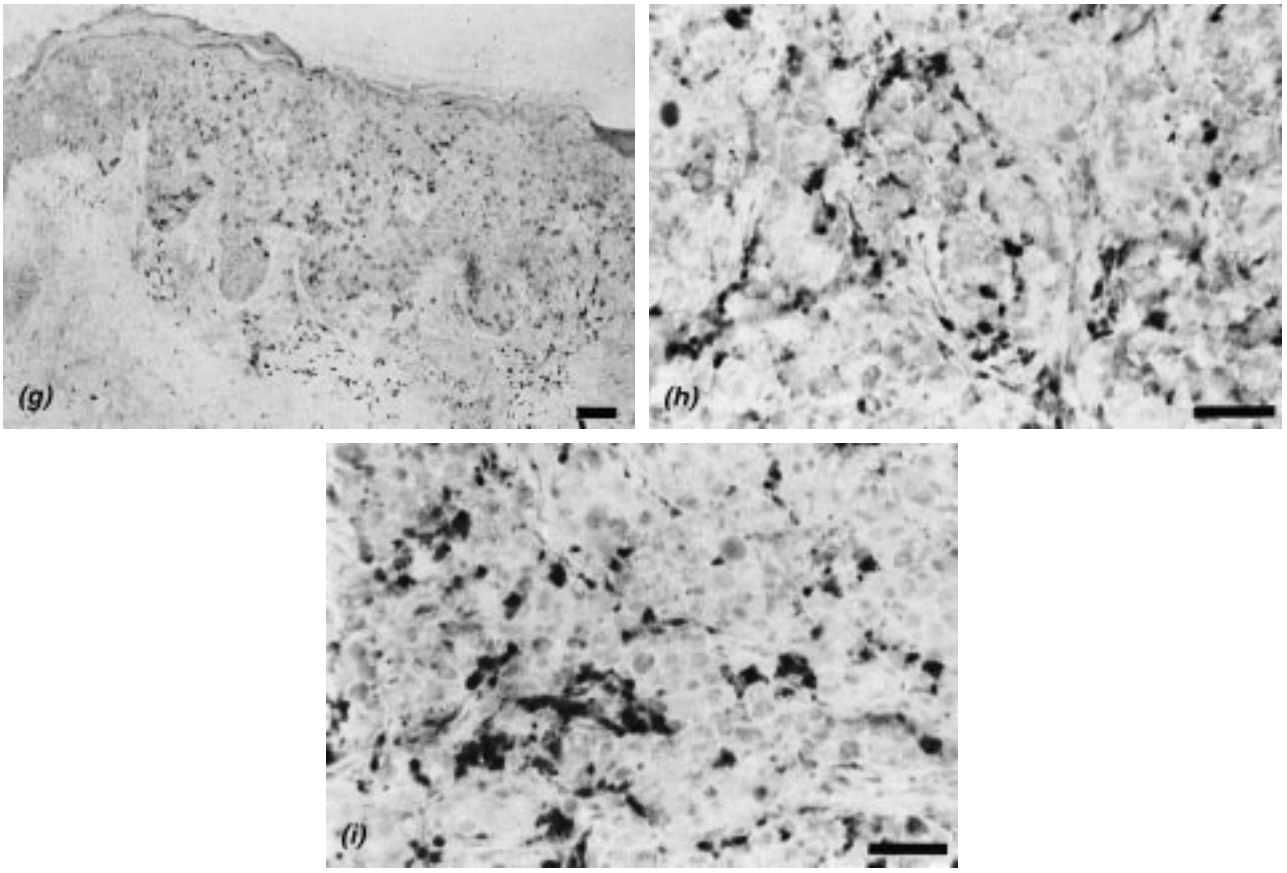

FIGURE 4 – Continued

also functions as a binding protein and not in growth factor signaling (Massagué, 1996). Nevertheless, most mammalian cells, including melanoma cells, express the TGF-βR type 1 and respond to the ligand (Rodeck *et al*., 1994). Normal cells are inhibited, whereas some metastatic cells are stimulated. The co-expression of ligand and receptor for GM-CSF appears to be significant biologically, but little else is known. The presence of the receptor on most cells of the majority of melanoma metastases should be taken into consideration in clinical studies when GM-CSF is used for immunostimulation. Based on our data, GM-CSF may also have melanoma-stimulating activities.

Besides potential autocrine roles, each cytokine may be secreted for paracrine stimulation of normal host cells in the environment. Normal fibroblasts may be involved, and TGF-β has been reported to increase matrix protein production in stimulated cells (Rizzino, 1988). IL-8 can act as an angiogenic factor (Koch *et al*., 1992) and is also a strong chemo-attractant for neutrophils (Ludwig *et al*., 1997). Thus, melanoma-derived IL-8 can exert diverse functions. TNF-α, TNF-β, IL-1, IL-6 and GM-CSF can all exert potent effects on the host inflammatory and immune cells. IL-6 is a potent activator of T and B cells (Paquet and Piérard, 1996), but it can also inhibit T-cell responses by inducing activation of TGF-β (Zhou *et al*., 1991), and GM-CSF stimulates anti-tumor immunity in murine models (Dranoff *et al*., 1993). Further experiments in model systems are required to better elucidate the activation pathways between inflammatory and immune cells, and between inflammatory and malignant cells.

SCFR is expressed by melanocytic cells, but expression decreases with progression, suggesting a decreasing need for the exogenous ligand in melanoma. Indeed, SCF is a potent mitogen for normal melanocytes (Grabbe *et al*., 1994), but its role in melanoma is less clear, because many malignant melanocytes have lower expression of both SCF and c-kit (Takahasi *et al*., 1995). SCF–SCFR interactions are important for neural crest cells during development and for the migration of these cells to the skin and differentiation to melanocytes (Fleischmann, 1993). Abnormalities in SCF expression lead to pigmentation disorders (Giebel and Spritz, 1991). In conclusion, our study is a comprehensive analysis of growth factor and cytokine expression patterns, and provides a baseline for mechanistic analyses of the biological function and significance of these molecules in melanoma.

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