Arch Dermatol Res (1995) 287:259–265

ORIGINAL PAPER

K. Krasagakis · C. Garbe · J. Eberle · C. E. Orfanos

Tumour necrosis factors and several interleukins inhibit the growth and modulate the antigen expression of normal human melanocytes in vitro

Received: 22 April 1994

Abstract In the present study the action of various cytokines as regulators of human melanocyte growth and differentiation was examined in vitro. Primary melanocyte cultures were obtained in complete medium free of 12-*O*-tetradecanoylphorbol-13-acetate or serum. First passage melanocytes were treated with various concentrations of recombinant tumour necrosis factor alpha and beta (rTNF-alpha, rTNF-beta), as well as with various recombinant interleukins (rIL-1a, rIL-1b, rIL-2, rIL-3, rIL-4 and rIL-6) for 6 days in complete medium and for 6 and 12 days in a mitogen-reduced medium variant. The 4-methylumbelliferyl heptanoate fluorometric microassay and Ki-67 staining were used for assessing cell proliferation, and the immunophenotype was evaluated using various monoclonal antibodies. Melanocyte proliferation in complete medium was inhibited by rTNF-alpha (–24%), rTNF-beta (–17%), rIL-1a (–21%), rIL-1b (–18%) and rIL-6 (–29%); in contrast, rIL-2, rIL-3 and rIL-4 had no antiproliferative effect. Measurements of Ki-67-positive nuclei confirmed these results. In the reduced medium variant, none of the above cytokines inhibited melanocyte proliferation. Recombinant TNF-alpha and rTNF-beta markedly reduced the expression of the pigment cell-associated antigens HMB-45 and K.1.2, and they enhanced the expression of VLA-2, ICAM-1 and HLA class I antigens and strongly induced HLA-DR. Similar changes were induced by rIL-1a, rIL-b and rIL-6, and rIL-2 decreased the expression of HLA class I antigens and of ICAM-1. In conclusion, several cytokines inhibited the growth and modulated the phenotype of melanocytes in vitro. Since these cytokines are major mediators of inflammatory processes, they may cause the pigmentary alterations seen after cutaneous inflammatory processes.

Key words Tumour necrosis factor · Interleukins · Cytokines · Melanocytes

Presented in part at the 23rd meeting of the European Society for Dermatological Research (ESDR). Amsterdam, 3–6 April 1992

K. Krasagakis (✉) · C. Garbe · J. Eberle · C. E. Orfanos
Department of Dermatology, University Medical Center Steglitz, The Free University of Berlin, Hindenburgdamm 30, D-12200 Berlin, Germany

Introduction

Inflammatory skin reactions may cause changes in skin pigmentation. It has been postulated that mediators present in inflamed skin participate in the control of melanocyte response (Nordlund et al. 1988). Cytokines have been implicated in the pathogenesis of atopic dermatitis (Thestrup-Pedersen et al. 1990; Furue et al. 1991) and in longer persisting skin diseases changes of skin colour have been observed. In vitiligo it has recently been suggested that an early inflammatory phase may occur leading to melanocyte destruction (Abdel-Nasar et al. 1994). Several studies have been performed on cytokines that positively regulate normal human melanocyte growth and differentiation in vitro, and the mitogens required for establishment of melanocyte cultures have been defined (Gilchrest et al. 1984; Halaban et al. 1987; Herlyn et al. 1990).

However, less information is available on the negative mechanisms controlling regulation of melanocyte growth and differentiation (Krasagakis et al. 1991; Swope et al. 1991). The aim of the present study was to explore any antiproliferative properties of tumour necrosis factors (TNFs) and the interleukin family on normal human melanocytes in vitro. Melanocyte proliferation was studied in the presence of cytokines in optimal and suboptimal mitogenic medium conditions. The modulation of several melanocyte antigens associated with activation (HMB-45), differentiation (K.1.2) and immune recognition (HLA class I antigens, HLA-DR, ICAM-1 and VLA-2) by the TNFs and the interleukin family were also studied.

Materials and methods

Melanocyte culture

Primary melanocyte cultures were obtained from trypsin-digested epidermis of foreskins in a 12-*O*-tetradecanoylphorbol-acetate (TPA)-free complete melanocyte medium (CMM). The CMM consisted of MCDB-153 (Biochrom, Berlin, Germany) supplemented with 2 μ*M* Ca^{2+}, 5 μg/ml insulin (Sigma, St. Louis, Mo., USA), 10 μg/ml human transferrin (Sigma), 0,4% v/v whole bovine pituitary extract (Clonetics, San Diego, Calif., USA), 2 ng/ml bovine basic fibroblast growth factor (Boehringer, Mannheim, Germany), 1 n*M* choleratoxin (Calbiochem, La Jolla, Calif., USA), 50 μ*M* hydrocortisone (Serva, Heidelberg, Germany) and antibiotics. For the first 3 days of primary culture 2% fetal calf serum (Seromed) was added and for the first 24 h of each subculture. Pure primary melanocyte cultures were obtained as shown by staining with S-100 antibodies using the alkaline phosphatase–antialkaline phosphatase method. A mitogen-reduced variant of the CMM without transferrin, basic fibroblast growth factor or choleratoxin, designated as reduced melanocyte medium (RMM), was used in some experiments. First or second passage melanocyte cultures were used for these studies.

Proliferation assays

First passage normal human melanocytes were seeded in tissue culture plates (96 flat-bottomed wells) (Becton Dickinson, Heidelberg, Germany) at 2×10^3 cells/well in 0.2 ml CMM supplemented with 2% fetal calf serum. The cultures were washed twice with CMM or RMM 1 day later and further incubated with fresh CMM or RMM respectively containing 0–80 ng/ml recombinant TNF-alpha (rTNF-alpha), 0–20 ng/ml rTNF-beta, 0–20 ng/ml recombinant interleukin 1 alpha (rIL-1a), 0–20 ng/ml rIL-1b, 0–80 ng/ml rIL-2, 0–80 ng/ml rIL-3, 0–40 ng/ml rIL-4, 0–40 ng/ml rIL-6 (British Biotechnology, Oxford, UK). Fresh medium containing the various cytokines was given every 2 days. Cell proliferation was determined on day 7 for CMM or on days 7 and 13 for RMM by a previously described fluorometric microassay using as substrate 4-methylumbelliferyl heptanoate (4-MUH; Sigma; Zouboulis et al. 1991). The fluorescence values measured using a Titertec Fluoroscan II (Flow Laboratories, Meckenheim, Germany) correlated well with cell counts determined independently.

Monoclonal antibodies

The modulation of the antigen expression of normal human melanocytes by cytokines in vitro was studied in CMM using the following, monoclonal antibodies (mAb): anti-Ki-67 (1:40; Dakopatts, Glostrup, Denmark) staining a nuclear antigen associated with proliferation; HMB-45 (1:6000; Enzo, N.Y., USA) as a pigment cell-specific marker detecting activated melanocytes; K.1.2 (1:2; Zelldiagnostika, Münster, Germany) staining a melanoma antigen negatively associated with malignancy; A.1.43 (1:20; Zelldiagnostika) as melanoma progression marker staining VLA-2; B 9.12.1 (1:100; Immunotech, Marseille, France) reacting with HLA class I antigens; L243 (1:40; Becton Dickinson, Mountain View, Calif) detecting HLA-DR class II antigens; and 84H10 (1:400; Immunotech) binding with the ICAM-1 molecule. All mAbs have been described elsewhere (Gerdes et al. 1983; Gown et al. 1986; Suter et al. 1985; Brüggen and Sorg 1983; Malissen et al. 1982; Lampson and Levy 1980; Rothlein et al. 1986).

Immunocytochemistry

Immunocytochemistry was performed using the alkaline phosphatase–antialkaline phosphatase (APAAP) method on acetone-fixed air-dried cytospin preparations of ethylenediamine tetraacetic acid-treated (0.5% in phosphate buffered saline, PBS) melanocyte cultures as previously described (Cordell et al. 1984). After incubation with the primary mouse mAb, a rabbit antimouse IgG (Dakopatts, Glostrup Denmark) and an antibody conjugated with alkaline phosphatase (Dianova, Hamburg, Germany) were applied. The slides were counterstained with Meyer's haematoxylin (1%). Negative controls were obtained by staining with isotype-matched irrelevant antibodies. On each slide, 200 cells were examined for reactivity with the mAb. The number of positive cells is given as the mean percentage of three independent experiments. The intensity of staining was graded on a scale of 0+ to 5+: 0+ no staining, 1+ minimal, 2+ mild, 3+ moderate, 4+ intense, and 5+ maximal staining.

Statistic

Proliferation experiments were performed five times and staining experiments three times; mean values and standard deviations were calculated. Statistical differences were evaluated by the two-sided Student's *t*-test.

Results

Effect of TNFs and interleukins on melanocyte proliferation

Proliferation studies were performed either in CMM or in RMM. Both rTNF-alpha and rTNF-beta inhibited melanocyte proliferation in a dose-dependent manner after 6 days of incubation (rTNF-alpha, 24% at 80 ng/ml, $P < 0.01$; rTNF-beta, 17% at 20 ng/ml, $P < 0.001$; Fig. 1A, B). Both rIL-1a and rIL-1b inhibited melanocyte proliferation in a dose-dependent manner in CMM (21% and 18% at 20 ng/ml, respectively, $P < 0.01$; Fig. 1C). Recombinant IL-2 (0–80 ng/ml), rIL-3 (0–80 ng/ml) and rIL-4 (0–40 ng/ml) had no effect on melanocyte proliferation after 6 days of incubation. In contrast, rIL-6 caused inhibition of melanocyte proliferation by up to 29% ($P < 0.001$ at 4 ng/ml; Fig. 1D).

In order to confirm these findings we produced cytospin preparations of cytokine-treated melanocytes and stained them with a mAb detecting the nuclear proliferation antigen Ki-67 using the APAAP technique. In accordance with the MUH measurements, a significant reduction in the number of positive nuclei was found after treatment with rTNF-alpha, rTNF-beta, rIL-1a, rIL-1b and rIL-6; in contrast, treatment with rIL-2, rIL-3 and rIL-4 had no effect (Fig. 2). Maximal reduction of staining was observed after treatment with 40 ng/ml rIL-6 and 80 ng/ml rTNF-alpha (22% and 19% positive nuclei) vs 51% for the controls ($P < 0.001$; Fig. 3).

We also investigated the effect of various cytokines on melanocytes cultured in RMM in order to exclude the possibility that 'superstimulated' melanocytes respond less to exogenously added cytokines, but none of the cytokines tested above was found to exert a significant effect on melanocyte proliferation after 6 and 12 days of incubation in RMM.

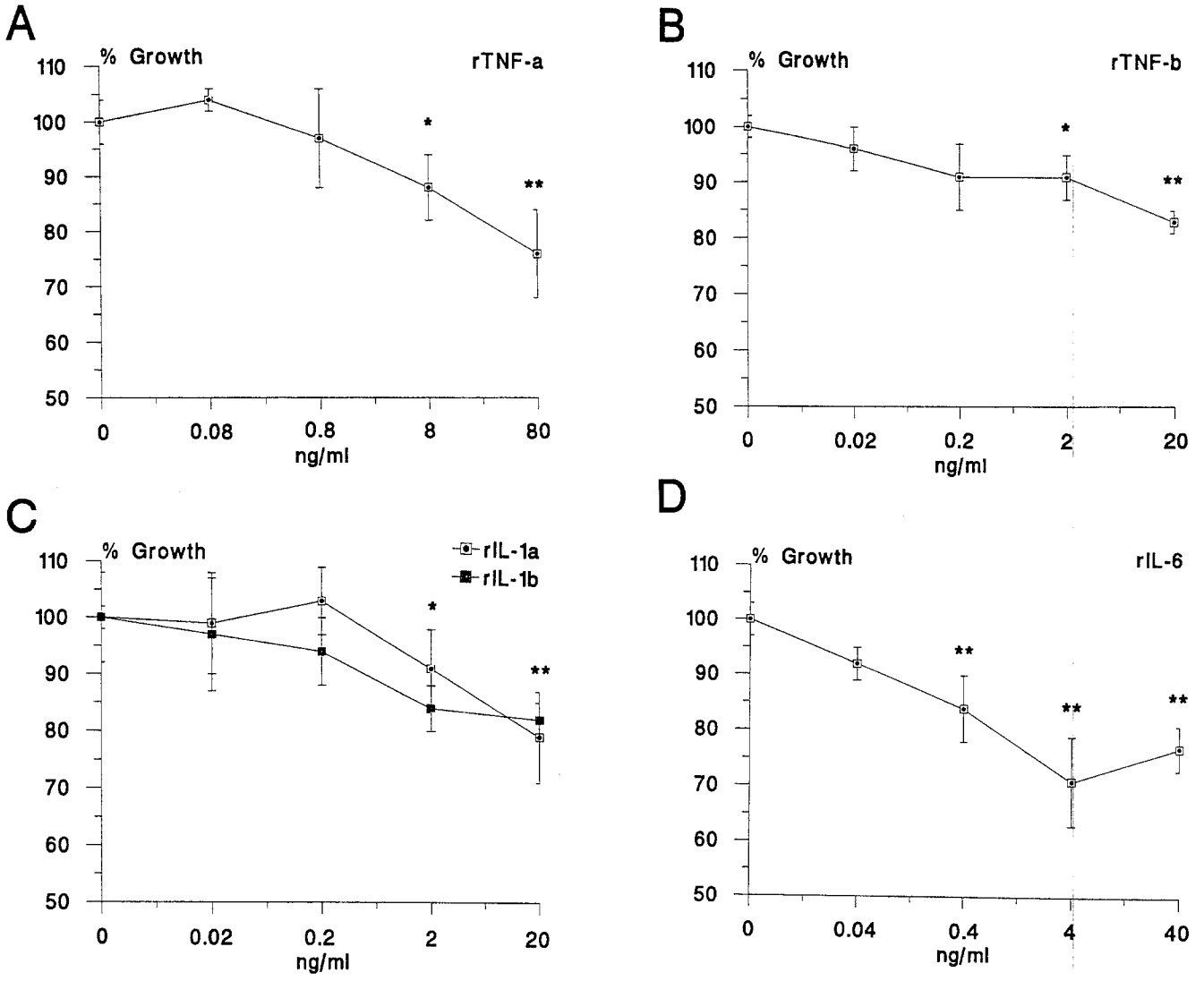

Fig. 1A–D Effect of human recombinant cytokines on melanocyte proliferation in vitro. First passage melanocyte cultures were incubated with various cytokines for 6 days in concentrations as indicated in "Materials and methods". Significant inhibition of proliferation was observed with rTNF-alpha (**A**), rTNF-beta (**B**), rIL-1a and rIL-1b (**C**), and rIL-6 (**D**) as shown in the MUH assay (* $P <$ 0.01, ** $P < 0.001$). Results of one representative experiment are illustrated

Modulation of the antigenic profile of melanocytes after treatment with TNFs or interleukins

Immunophenotypic examination of melanocytes for modulation of antigen expression was performed in CMM. In this medium the melanocyte phenotype showed 100% positivity for the activation antigen HMB-45 as well as for the differentiation marker K.1.2 and the HLA class I antigens. The adhesion receptor VLA-2 stained by the mAb A.1.43 was faintly expressed in 15% of the cells. Mild staining was found in 80% of the cells for ICAM-1, but no staining for HLA-DR.

Interestingly, the cytokines that inhibited melanocyte growth also induced antigenic alterations on melanocytes

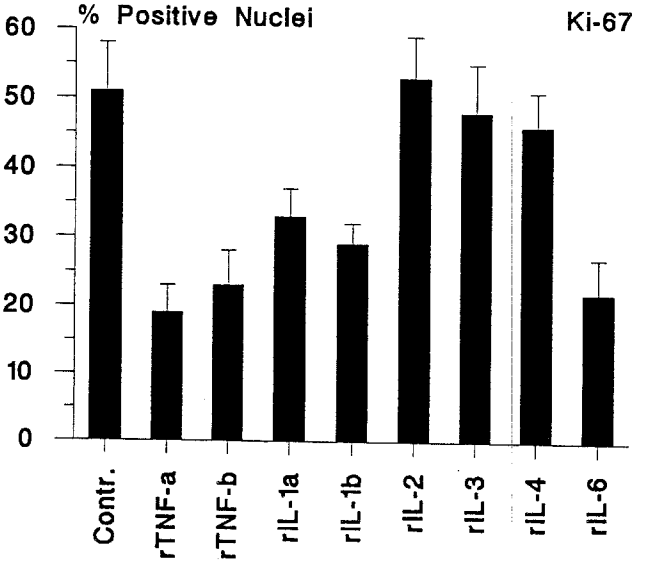

Fig. 2 Influence of TNFs and interleukins on Ki-67 expression. Melanocytes were incubated for 6 days with various cytokines and stained with a mAb against Ki-67 nuclear antigen. A significant reduction in the number of Ki-67$^+$ nuclei was found after treatment with rTNF-alpha, rTNF-beta, rIL-1a, rIL-1b and rIL-6

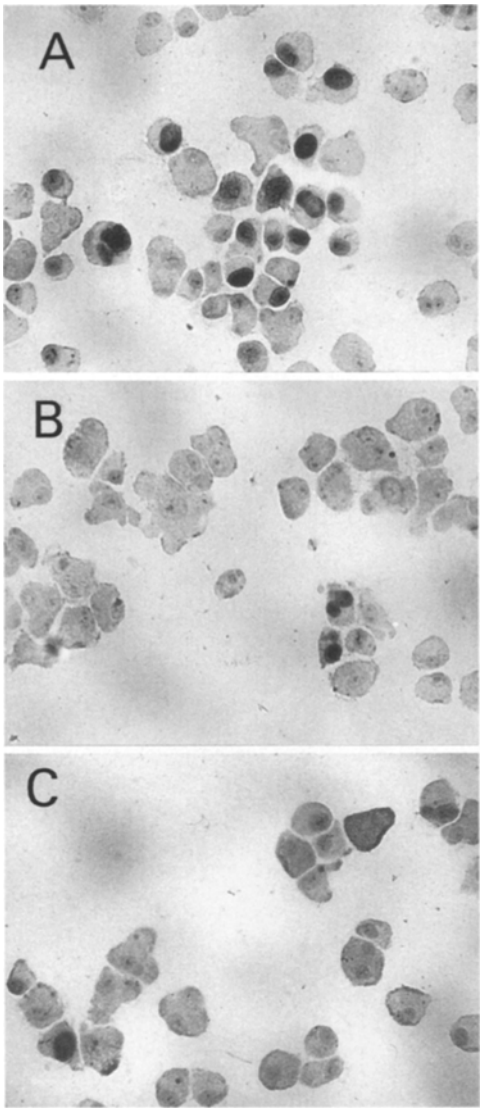

Fig. 3A–C Cytospin preparations of melanocyte cultures untreated (**A**), or treated with 80 ng/ml rTNF-alpha (**B**) or 40 ng/ml rIL-6 (**C**) and stained with a mAb against Ki-67 using the APAAP technique (× 500)

(Table 1). The cytokines of the TNF family and especially rTNF-alpha modulated the expression of all antigens examined (Table 1; Figs. 4 and 5). The staining intensity with the mAb against activation antigen HMB-45 as well as with the differentiation marker K.1.2 was substantially decreased from 4+ to 2+ after treatment with rTNF-alpha or rTNF-beta (Table 1). In addition, rTNF-alpha decreased the number of K.1.2$^+$ melanocytes from 100% to 78% (Fig. 5). Significant modulation of VLA-2 adhesion receptor was seen after incubation with rTNF-alpha or rTNF-beta as stained with the progression marker A.1.43 (71% and 32% positive cells, respectively) vs 15% for the controls ($P < 0.001$; Fig. 4). The staining intensity for HLA class I was increased after treatment with rTNF-alpha and rTNF-beta (Table 1). Both cytokines upregulated ICAM-1 expression (100% positive cells, $P < 0.001$) and induced expression of HLA-DR (22% and 8% positive cells with rTNF-alpha and rTNF-beta, respectively, $P < 0.001$; Figs. 4 and 5).

Similar antigenic changes were induced by several interleukins. Recombinant IL-1a and rIL-1b reduced the staining intensity with the differentiation marker K.1.2 and upregulated HLA class I antigens (Table 1). Recombinant IL-1a, rIL-1b as well as rIL-6 increased both the staining intensity and the number of positive melanocytes with ICAM-1 and these cytokines enhanced the expression on more than 94% of the cells ($P < 0.01$; Table 1 and Fig. 4). In a similar manner to ICAM-1, induction of HLA-DR antigen was seen after incubation of melanocytes with rIL-1a, rIL-1b, and rIL-6, and rIL-6 was found to be the most potent induced of HLA-DR (27% positive cells at 40 ng/ml; Fig. 4). In contrast to the other cytokines, rIL-2 substantially reduced the staining intensity of melanocytes for HLA class I antigens from 3+ to 1+ (Table 1) and decreased the number of ICAM–1$^+$ melanocytes from 81% to 59% ($P < 0.001$; Fig. 4). The cytokines rIL-3 and rIL-4 had no effect on the antigen expression of human melanocytes.

Discussion

The findings presented here provide a detailed characterization of the antiproliferative and immunomodulatory effects of a variety of cytokines of the TNF and interleukin families on cultured melanocytes. The results extend previous observations by us and others that growth and differentiation of melanocytes, non-lymphoid cells, are

Table 1 Modulation by interleukins and TNFs of the intensity of staining by mAbs of cultured human melanocytes in vitro. Values are staining intensity graded on a scale from 0 to 5+: 0, no staining; 1+, minimal; 2+, mild; 3+, moderate; 4+, intense; 5+, maximal staining (*NC*, no change in comparison to control)

Cytokine	mAb (antigen)					
	HMB-45	K.1.2	A.1.43	HLA class I	HLA-DR	ICAM-1
Controls	4+	4+	1+	3+	0	2+
rIL-1a	NC	2+	NC	4+	3+	3+
rIL-1b	NC	2+	NC	4+	3+	3+
rIL-2	NC	NC	NC	1+	NC	1+
rIL-3	NC	NC	NC	NC	NC	NC
ril-4	NC	NC	NC	NC	NC	NC
rIL-6	NC	NC	NC	NC	3+	3+
rTNF-alpha	2+	2+	2+	4+	3+	4+
rTNF-beta	2+	2+	2+	4+	3+	3+

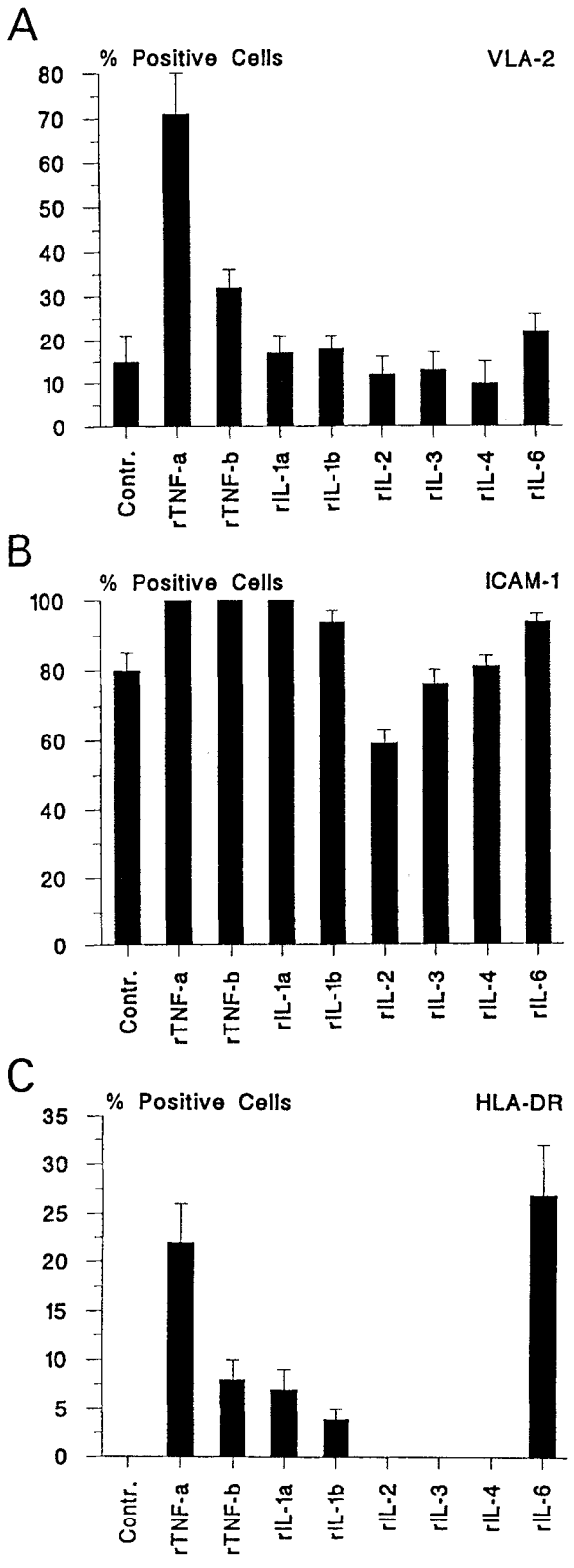

Fig. 4A–C Modulation of melanocyte antigen expression by cytokines in vitro. First passage melanocyte cultures were incubated with various cytokines for 6 days at concentrations as indicated in "Materials and methods". Cytospin preparations were made and stained using the APAAP technique. Results of one experiment performed in triplicate at highest cytokine concentrations are shown. A common pattern of modulation was observed showing upregulation of adhesion molecules VLA-2 (**A**), ICAM-1 (**B**) as well as of HLA-DR (**C**) ($n = 3$)

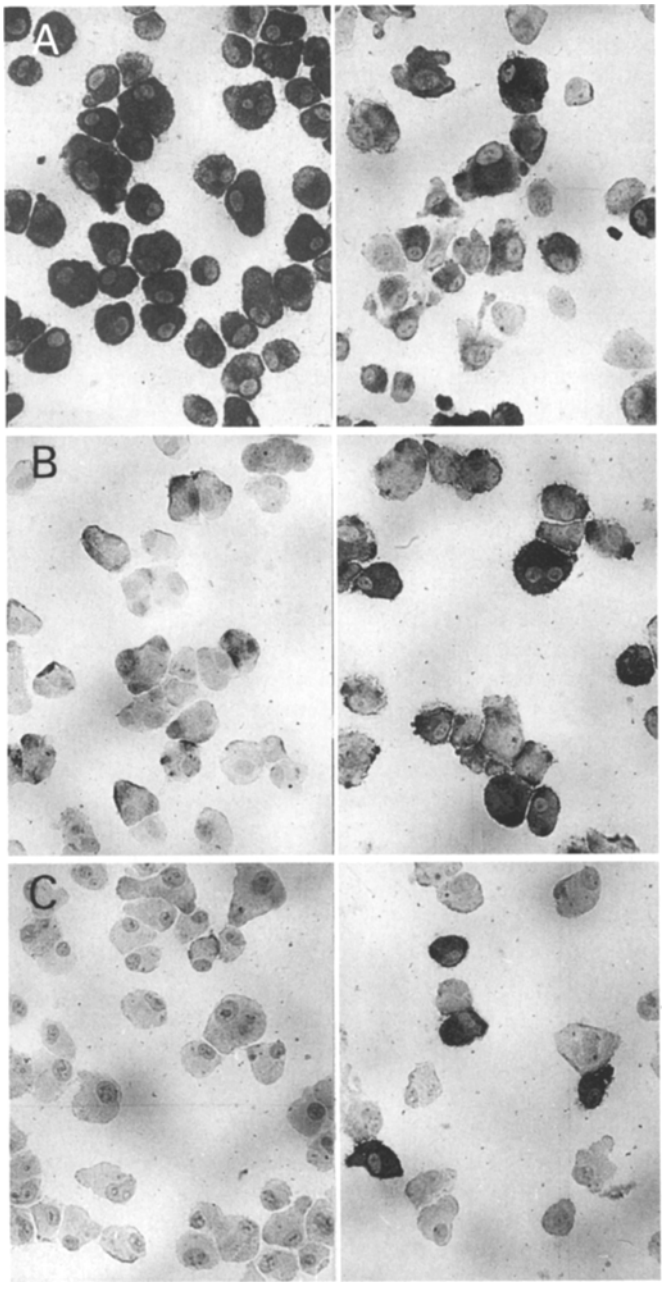

Fig. 5A–C Effect of rTNF-alpha on melanocyte antigen expression in vitro. APAAP staining of cytospins of melanocyte cultures with K.1.2 (**A**), ICAM-1 (**B**) and HLA-DR (**C**) after 6 days of incubation with 0 *(left)* or 80 ng/ml rTNF-alpha *(right)* is shown (× 500)

partly regulated by lymphokines in vitro (Yohn et al. 1990; Krasagakis et al. 1991; Swope et al. 1991). These findings should viewed not only together with the results of recent investigations demonstration the capacity of melanocytes to produce several immunoregulatory cytokines in vitro (Krüger-Krasagakes et al. 1994), but also considering the fact that substantial amounts of cytokines are relased by non-melanocytic cells, i.e. keratinocytes and lymphocytes, in inflammatory skin diseases (Ansel et al.

1990; Bochner et al. 1990; Thestrup-Pedersen et al. 1990). We have shown here that these cytokines are also involved in the regulation of the surrounding melanocytic cells.

The present study demonstrates for the first time that melanocytes are inhibited by rTNF-beta and rIL-1b. We also found that melanocyte growth is inhibited by rTNF-alpha, rIL-1a and rIL-6, and confirmed the observations of Swope et al. (1991). In our series of experiments rIL-6 was also inhibitory in the absence of TPA. Since previous investigations examining the effects of interferons on melanocytic cells have shown that 'superstimulated' melanocytes may only partially respond to cytokines (Krasagakis et al. 1991), we examined the effect of TNFs and interleukins in both complete (CMM) and in reduced (RMM) melanocyte media allowing high and low replication rates, respectively. As for interferons, a most appropriate medium for demonstrating the effect of cytokines was CMM. For investigating the effect of cytokines on melanocyte proliferation we also studied the presence of Ki-67, a nuclear cell cycle antigen whose expression correlates with cell proliferation (Gerdes et al. 1983). A decreased number of Ki-67-positive nuclei was seen after treatment with rTNF-alpha, rTNF-beta, rIL-1a, rIL-1b and rIL-6, supporting our measurements using the MUH assay. Interestingly, rTNF-alpha also inhibits proliferation of epidermal keratinocytes (Detmar and Orfanos 1990). In contrast to our findings on melanocytes, other skin cell populations including keratinocytes, dermal microvascular endothelial cells and fibroblasts are stimulated by IL-1 (Sauder et al. 1988; Detmar et al. 1992; Schmidt et al. 1982). Thus, these findings point to a differential control of proliferation of melanocytes by IL-1 among cutaneous cell populations.

Antigen expression

To date, only few experimental data on the modulation of melanocyte antigens by interleukins and TNFs have been reported (Yohn et al. 1990; Kirnbauer et al. 1992). HMB-45 has been ultrastructurally localized in melanosomes (stages 1–3; Schaumburg-Lever et al. 1991) and has been regarded as an activation marker for melanocytes (Smoller et al. 1990). The K.1.2.-defined antigen, on the other hand, can be seen as a melanocyte differentiation marker, and its expression decreases during melanoma progression (Suter et al. 1985). Modulation of the VLA-2 adhesion molecule was studied by staining with the melanoma progression marker A.1.43 (Brüggen and Sorg 1983; Klein et al. 1991). Several antigens involved in immune recognition were also investigated, such as HLA class I antigens, HLA-DR and ICAM-1.

The findings presented here indicate that TNF-alpha and TNF-beta act as potent immune regulators of melanocytes. They lowered the intensity of HMB-45 and downregulated the K.1.2 antigen. These alterations possibly reflect a decreased state of activation and differentiation of the melanocytes. VLA-2 was enhanced by treatment with rTNF-alpha and rTNF-beta. In accordance with the results of previous investigations with interferons (Tsujisaki et al. 1987; Krasagakis et al. 1991), both rTNF-alpha and rTNF-beta enhanced HLA class I and induced HLA-DR expression. The modulation of melanocyte ICAM-1 by immune cytokines has previously been extensively studied and our finding of its upregulation by rTNF-alpha and rTNF-beta (Yohn et al. 1990; Kirnbauer et al. 1992) were confirmatory.

Recombinant IL-1a, rIL-1b and rIL-6 modulated the immunophenotype of cultured melanocytes: rIL-1a and rIL-1b reduced the expression of the K.1.2 antigen and enhanced the expression of HLA class I, and rIL-6 potently induced HLA-DR (27% positive cells at 40 ng/ml) together with rIL-1a and rIL-1b. These cytokines also enhanced ICAM-1 expression on melanocytes. Similar findings on the modulation of ICAM-1 by interleukins have been previously reported for endothelial cells with the exception of IL-6 which had no effect on this cell population (Detmar et al. 1992). In contrast, rIL-2 decreased the expression of ICAM-1 and HLA class I antigens on cultured melanocytes. The downregulation of melanocyte ICAM-1 is novel and rather surprising since Kirnbauer et al. (1992) found no modulation by rIL-2. In their experiments, however, untreated melanocytes expressed no or minimal amounts of ICAM-1. In support of our observations in melanocytes, IL-2 also decreases the expression of HLA class I and of ICAM-1 in melanoma cell lines (Plaisance et al. 1993).

In conclusion, our studies revealed the rTNFs as well as rIL-1a, rIL-1b and rIL-6 inhibit the growth of melanocytes and alter their immunophenoype, whereas rIL-2 influences only the immunophenotype. The melanocyte population observed after cytokine treatment, showing low HMB-45, decreased K.1.2 and enhanced or new expression of ICAM-1, HLA class I and HLA-DR, may represent a less differentiated phenotype of melanocytic cell. This is further supported by the observations of Swope et al. (1991) who showed decreased tyrosinase activity of melanocytes treated with IL-1a, IL-6 or TNF-alpha. Since IL-1 and IL-6 as well as TNF-alpha have been detected in atopic and contact dermatitis (Bochner et al. 1990; Thestrup-Pedersen et al. 1990) and the expression of ICAM-1 and HLA-DR was found on melanocytes (Al Badri et al. 1993), we believe that various cytokines may play a role in vitiligo and may contribute to skin hypopigmentation. The presence of cytokines like TNFs or interleukins in vitiligo lesions of other postinflammatory hypopigmentations remains to be investigated in detail.

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