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ORIGINAL ARTICLE

Alexei F. Kirkin · Per thor Straten · Jesper Zeuthen

Differential modulation by interferon γ of the sensitivity of human melanoma cells to cytolytic T cell clones that recognize differentiation or progression antigens

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Abstract Human melanoma is a highly immunogenic tumor capable of inducing a specific immune response. A number of melanoma-associated antigens have been characterized during the past several years and can be classified into two groups: differentiation antigens – present also in normal melanocytes – and tumor-specific antigens, which, with the exception of testis, are present only in tumor cells. In a previous publication [Kirkin A. F., Petersen T. R., Olsen A. C., Li L., thor Straten P., Zeuthen J. (1995) Cancer Immunol Immunother 41:71] we have described the production of clones of cytotoxic T lymphocytes (CTL) against the highly immunogenic human melanoma cell line FM3. Using these clones we have defined four previously unknown melanoma-associated antigens, which could be subdivided into differentiation and progression antigens. In the experiments reported in this paper, we have further compared CTL clones from different groups and shown that the sensitivity of melanoma cells to CTL that recognize differentiation or progression antigens is differentially modulated during tumor progression as well as by the lymphokines interferon γ (IFNγ) and interleukin-10 (IL-10). The interaction of CTL clones recognizing progression antigens was strongly increased after treatment of melanoma cells with IFNγ, while the recognition by CTL clones specific for differentiation antigens either was unchanged or significantly decreased. IL-10 treatment of melanoma cells induced up-regulation with respect to recognition by CTL clones specific for differentiation antigens without affecting the recognition of melanoma cells by CTL clones specific for progression antigens. Using cellular systems at different stages of tumor progression, we demonstrated that the progressed state of melanoma cells is associated with increased sensitivity to recognition by CTL clones detecting progression antigens, and with decreased sensitivity to CTL clones recognizing differentiation antigens. Mimicking tumor progression, treatment with IFN-γ induced apparent down-regulation of differentiation antigens. A hypothesis is suggested in which IFN-γ plays different roles in the immune response against poorly immunogenic and highly immunogenic melanoma cells, increasing the progression of poorly immunogenic tumor cells or promoting a strong immune response and regression of highly immunogenic melanoma cells.

Key words Melanoma · Melanoma-associated antigens · Cytolytic T cell clones · Interferon γ · Interleukin-10 · Immunogenicity

A. F. Kirkin · P. thor Straten · J. Zeuthen (✉)
Department of Tumor Cell Biology, Division of Cancer Biology, Danish Cancer Society, Strandboulevarden 49, DK-2100 Copenhagen, Denmark

Fax: +45 35 25 77 21; e-mail: JZ@kbbio.kb.inst.dk

Introduction

One of the main goals of tumor immunology is the characterization of components of the specific antitumor immune response. It has long been suggested that tumor cells contain tumor-specific rejection antigens, which can induce activation of specific T cells and initiate the development of the cytotoxic response against tumor cells (see review [11]). During the last few years, several tumor-associated rejection antigens have been characterized, mainly in human melanoma cells. These antigens belong to two different classes. The first class consists of the so-called differentiation antigens present in both melanoma cells and normal melanocytes. Among such antigens are tyrosinase [5, 41], tyrosinase-related protein, gp75 [39], gp100/Pmel17 [2, 15] and Melan-A/MART-1 [6, 16]. The second class consists of antigens expressed in tumors in a very specific fashion. Examples of such antigens in humans are MAGE-1 [36], MAGE-3 [10], BAGE [3] and GAGE-1/2 [35]. Normal cells from adult tissues, with the exception of testis, do not express these antigens while some other tumors may express these antigens. It is noteworthy that cytotoxic T lymphocytes showing specificity for differentiation antigens were repeatedly obtained during cultivation of patient lymphocytes in the presence of melanoma cells

and interleukin-2 [29]. On the other hand, cytotoxic T lymphocytes specific for antigens of the second class were obtained only from the lymphocytes of one particular patient, patient MZ2, who was subjected to multiple immunizations with syngeneic, mutagen-modified melanoma cells [34]. The reasons for these differences are not known, but it is evident that characterization of the antigens that can induce specific antitumor immune responses without affecting normal tissues is of high priority.

In a previous paper [17], we have described the production of cytotoxic T lymphocyte (CTL) clones against the HLA-A2$^+$ highly immunogenic melanoma cell line FM3. These clones, according to their pattern of reactivity with different HLA-A2$^+$ melanoma cell lines, were classified into four antigen-recognition groups. CTL clones of only one of these groups (group D) recognized normal melanocytes, pointing to the involvement of some lineage-specific differentiation antigen. Two other groups of CTL clones (groups B and C) recognized several melanoma cell lines, indicating the recognition of shared tumor-associated antigens. The fourth group (group A), recognizing only FM3 cells, resembles unique tumor-specific rejection antigens similar to antigens described previously for some experimentally induced tumors [27] and also to the recently described unique melanoma-associated antigens MUM-1 [7] and mutated CDK4 [42]. In this paper, we present additional evidence for the definition of some melanoma-associated antigens as progression antigens, and also show that the sensitivity of target cells to CTL clones recognizing differentiation and progression antigens is differentially modulated after treatment with interferon γ (IFNγ) or interleukin-10 (IL-10).

Materials and methods

Cell lines

The establishment and properties of melanoma cell lines FM3, FM28, FM45, FM48, FM55$_P$, FM55$_P$.R22, FM55$_{M1}$, FM55$_{M2}$, FM59 and FM60 have been described previously [17]. The FM3.D variant of FM3 cells was isolated upon a second attempt to establish a cell line from the original biopsy material by isolation at early passages of subculture of cells having dendritic morphology. In contrast to the isolation of FM3 cells NIH3T3 feeder cells were not used. The melanoma cell lines SK29-Mel and LB373-Mel and the Epstein-Barr-virus(EBV)-transformed B-lymphoblastoid cell lines Alex K-EBV and LG2-EBV were kindly provided by Dr P. van der Bruggen, Ludwig Institute for Cancer Research, Brussels, Belgium. The B-lymphoblastoid cell line CALOGERO (HLA-A2,B61,C2,DR16,DQ5, DP4) from the 9th and 10th Histocompatibility Antigen Workshops) was obtained from the Tissue Typing Laboratory, Copenhagen University Hospital. Cells were cultured in RPMI-1640 medium (Gibco Laboratories, Grand Island, N.Y., USA) containing 10 mM HEPES buffer, penicillin, streptomycin and 10% fetal calf serum (FCS). EBV-transformed B cell lines were cultured in the same medium. Generation of autologous CTL clones against the melanoma cell line FM3 was described previously [17]. One additional CTL clone with characteristics of CTL group D (CTL clone 33) was also used in this study. CTL clones were repeatedly restimulated with FM3 melanoma cells as stimulator cells and CALOGERO cells as feeder cells as described previously [17], with the exception that pooled human serum was used instead of FCS. The CTL clones IVSB (anti-tyrosinase) and 246/15 (anti-Melan-A) were kindly provided by Dr. P. van der Bruggen, Ludwig Institute for Cancer Research, Brussels, Belgium, and were cultured by weekly restimulation with irradiated SK29.1-Mel and Alex K-EBV cells (clone IVSB) or LB373-Mel and LG2-EBV cells (clone 246/15).

Cytotoxicity assays

Samples containing 10^6 target cells were labelled for 90 min at 37 °C with 100 μCi Na_2CrO_4 (Amersham, UK). After being washed twice with cold RPMI-1640 medium, the targets were adjusted to a concentration at 5×10^4 cells/ml in RPMI-1640 medium with 10% FCS. The effector cells were resuspended at various concentrations. Effector and target cells were seeded in 100-μl aliquots in 96-well U-bottomed microtiter plates, spun at 60 *g* for 5 min and incubated at 37 °C in 5% CO_2. After 4 h, 100 μl supernatant was harvested and the radioactivity was determined (Cobra 5005, Packard Instruments, Meriden, Conn., USA). The specific lysis was calculated according to the following equation:

$$\text{Specificlysis}(\%) = \frac{\text{experimental release(cpm)} - \text{spontaneous release(cpm)}}{\text{maximum release(cpm)} - \text{spontaneous release(cpm)}} \times 100$$

where the spontaneous release was determined by incubating target cells in the absence of effectors and the maximum release was determined in the presence of 1% sodium dodecyl sulfate. Spontaneous lysis did not exeeded 7%–8%. The treatment of melanoma cells with human recombinant IFNγ, IL-4, IL-10 (all from Genzyme, Boston, USA) or tumor necrosis factor α (TNFα; a gift from Dr. Marja Jäättelä, Copenhagen) was done during 3–6 days starting the day after cells were seeded into tissue flasks, as described in the text and legends to the figures. All the experiments on the cytotoxic activity of CTL clones were repeated at least three times and the results of one representative experiment are shown.

Antibodies and flow-cytometric analysis

Unconjugated, fluorescein-isothiocyanate(FITC)- or phycoerythrin-conjugated monoclonal antibodies against ICAM-1, LFA-3, BB1/B7 and HLA-DR were purchased from Becton Dickinson (Mountain View, Calif., USA). The monoclonal antibodies W6/32, MA2.1 and PA2.1 were purified from hybridoma supernatants using a protein-A-–Sepharose-4B column (Pharmacia, Uppsala, Sweden). For indirect immunofluorescence staining, a goat anti-mouse FITC-labelled F(ab′)$_2$ antibody was used as the secondary antibody. Flow-cytometric analyses were carried out using a FACScan flow cytometer (Becton Dickinson, Immunocytometry Systems, San Jose, Calif., USA).

Tumor growth in nude mice

BALB/c strain male nude (nu/nu) mice were maintained under specific-pathogen-free conditions. Untreated or IFNγ-treated melanoma cells, FM3 and FM3.D (5×10^6 cells), were injected subcutaneously in 0.2 ml phosphate-buffered daline.

Reverse-transcription(RT)-coupled polymerase chain reaction (PCR)

RNA was isolated from FM3, FM3 treated with INFγ, FM3-D and FM3-D treated with INFγ. The protocol for RNA isolation has previously been described [17]. Total RNA was measured at $A_{260/280}$ and oligo(dT)-primed complementary DNA (cDNA) was synthesized using 2 μg RNA, and 200 U reverse transcriptase (M-MLV, Life Technologies) following the manufacturer's suggestions. After 60 min incubation at 37 °C and a 5-min inactivation at 99 °C, eight serial dilutions (1/5) of each cDNA preparation were prepared and PCR performed with primers for the glyceraldehyde phosphate dehydrogenase gene (*GAPDH*) for 35 cycles. Aliquots of 10 μl were run in agarose

Table 1 Primer sequences and product sizes. *GAPDH* glyceraldehyde-phosphate dehydrogenase

Gene	Primer	Sequence	Product size (bp)
GAPDH	Sense	5′-AGGGGGGAGCCAAAAGGG-3′	540
	Anti-sense	5′-GAGGAGTGGGTGTCGCTGTTG-3′	
Melan-A	Sense	5′-GAAGGTGTCCTGTGCCCTGACCC-3′	497
	Anti-sense	5′-GGCTTGCATTTTTCCTACACCATTCC-3′	
MAGE-3	Sense	5′-ACCAGAGGCCCCCGGAGGAG-3′	535
	Anti-sense	5′-CTGCCAATTTCCGACGACACTCC-3′	
Tyrosinase	Sense	5′-CAGCACCCCACAAATCCTAAC-3′	417
	Anti-sense	5′-CAACAAATGCATGGTGAAGAAGG-3′	
gp100	Sense	5′-GGCTGGTGAAGAGACAAGTCC-3′	469
	Anti-sense	5′-AGAGATGCAAGGACCACAGCC-3′	

gels and stained with ethidium bromide. Based on the intensity of the *GAPDH* PCR products in the gel, six suitable cDNA dilutions were selected for the analysis of the expression of the genes for the known melanoma-associated antigens tyrosinase, MART-1/Melan-A, MAGE-3 and gp100. In order to be able to work on precisely the same amount of cDNA in each reaction, all reactions were set up using a master mix for the particular cDNA. The master mix was split between each of the different reactions after thorough mixing, and reactions were carried out at least twice.

The analysis was carried out to investigate differences in gene expression regarding the transcripts for the MAGE-3, gp100, MART-1/Melan-A and tyrosinase genes using *GAPDH* as control. The primer sequences used and the size of the amplified cDNA are shown in Table 1. Optimal primer sequences were found using the computer program Oligo 3.4 (Medprobe, Norway). Reactions were carried out in 25 µl 1×PCR buffer [50 mM KCl, 20 mM TRIS pH 8.4, 2.5 mM $MgCl_2$, 0.005% (w/v) bovine serum albumin (Boehringer-Mannheim, Mannheim, Germany)] containing 5 pmol each primer, 2.5 µM dNTPs (Pharmacia LKB, Uppsala, Sweden) and 0.5 unit Supertaq polymerase (HT Biotechnology Ltd., Cambridge, UK). The parameters for the amplification were 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. A "hot start" procedure was used in which Taq polymerase and dNTPs were added to the reaction tube at an 80 °C step between the denaturation and annealing steps of the first cycle. Amplifications were performed with a Hybaid Omnigene Thermal Cycler (Hybaid, Teddington, Middlesex, UK) using standard microtubes (Mµlti Technology Inc., Salt Lake City, Utah, USA). For quantification, aliquots of the PCR reactions were electrophoresed through 2% NuSieve agarose containing ethidium bromide, scanned under UV light and saved as a computer file. Quantification was carried out using Molecular Dynamics ImageQuant software [14]. The expression of the MART-1, tyrosinase, gp100 and MAGE-3 genes was determined by quantifying the specific PCR products and comparing these to the quantitation of the PCR product derived from the amplification of *GAPDH*.

Results

Recognition of FM3 cells with different states of tumor progression by CTL clones

In a previous paper [17], we have compared the recognition of primary and metastatic tumor cells from patient 55 by the CTL clones 22, 32 and 47. While all these clones recognized both primary and metastatic cells, the recognition of metastatic cells by clone 47 was higher as compared to the recognition of the same cells by clones 22 and 32. This fact was interpreted as an indication of the association of antigens recognized by CTL clone 47 with the state of progression of melanoma cells. In order to obtain additional evidence for this interpretation of the data, we decided to establish different subcultures of FM3 cells at different states of progression. From the original biopsy material, we isolated a subculture of cells that showed a more differentiated phenotype. The isolation was performed at early passages and without NIH3T3 fibroblasts as feeder

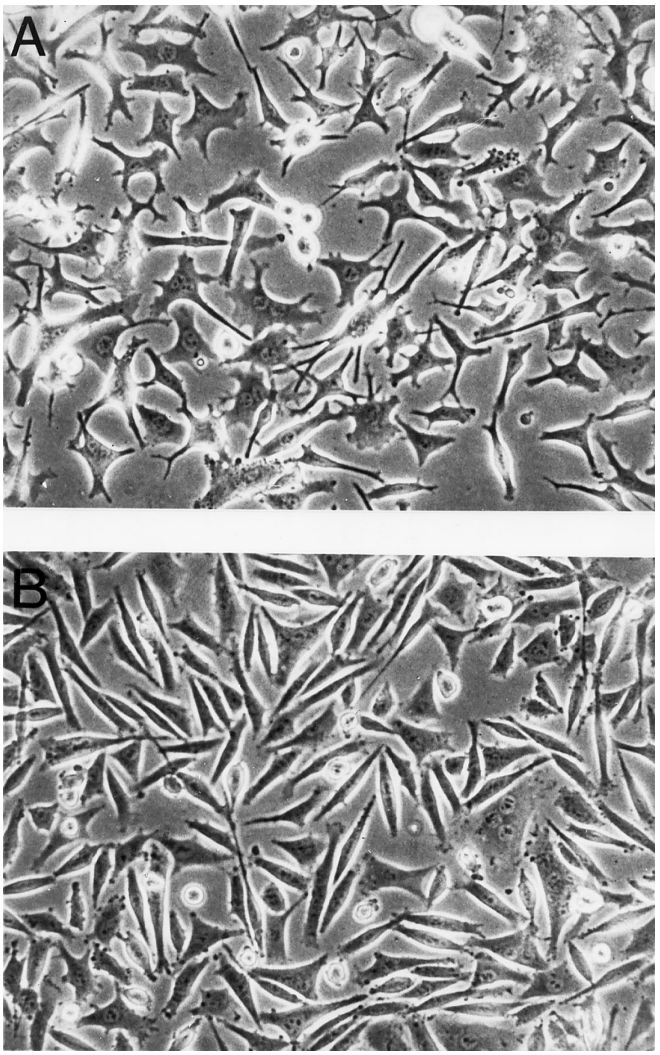

Fig. 1A,B Morphology of FM3.D (**A**) and FM3 (**B**) cells

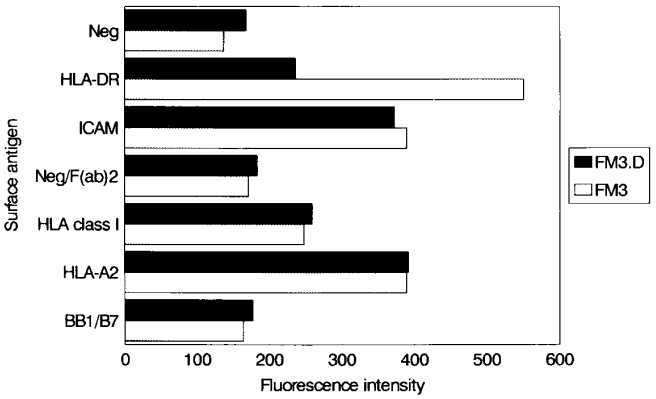

Fig. 2 Expression of surface markers by FM3.D (*black bars*) and FM3 (*hatched bars*) cells

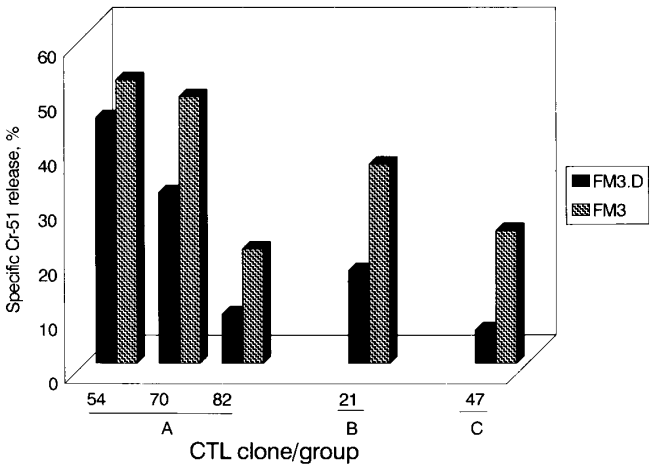

Fig. 3 Lytic activity (percentage specific ^{51}Cr release) of cytotoxic T lymphocyte (*CTL*) clones from different antigen-recognition groups (*A, B* and *C*) on FM3.D cells (*black bars*) and FM3 cells (*hatched bars*). The CTL:tumor cell(TC) ratio was 10

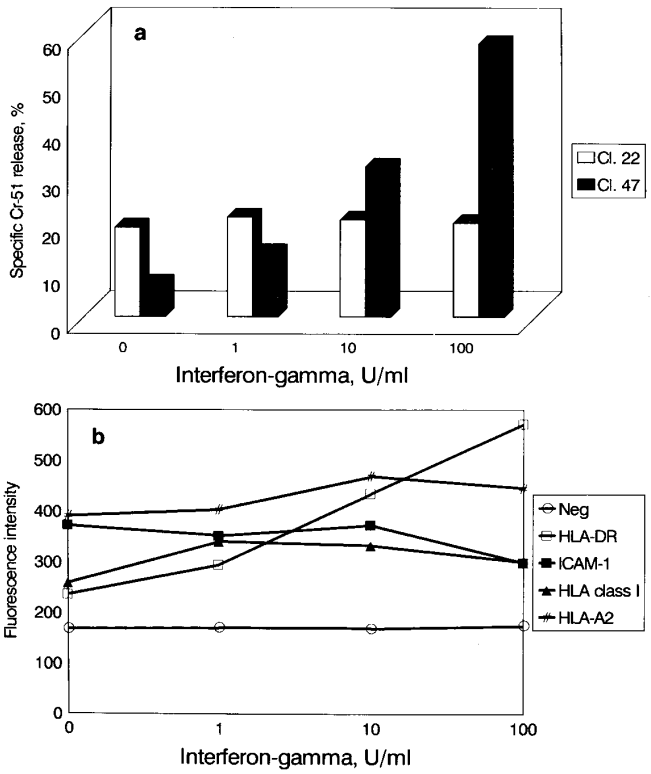

Fig. 4a,b Modulation of the properties of FM3.D cells after treatment with different concentrations of interferon γ (IFNγ). Melanoma cells were treated for 3 days. **a** Lysis of melanoma cells by CTL clones 22 and 47. The CTL:TC ratio was 10. **b** Expression of surface markers

cells, which had been used for the establishment of the previous FM3 cell line [25]. The new cell line was designated FM3.D. A comparison of the properties of FM3.D and FM3 cells is given in Figs. 1 and 2. Morphologically, FM3.D cells showed a predominantly dendritic appearance, while FM3 cells appeared to exhibit a less differentiated phenotype (Fig. 1). The expression of MHC class II antigens was significantly higher with FM3 cells than with FM3.D cells (Fig. 2). The melanin content was higher for FM3.D cells (data not shown). In addition, FM3 cells proliferated more rapidly (data not shown). All these differences indicate the presence of a more malignant phenotype in FM3 cells compared to FM3.D cells (e.g. [12]). Therefore, these cultures were judged to represent cultures at different states of tumor progression.

The interaction of CTL clones from the antigen-recognition groups A, B and C with these two different types of melanoma cells is presented in Fig. 3. The sensitivity of FM3 cells to CTL clones of the groups A (CTL clones 54, 70 and 82), B (CTL clone 21) and C (CTL clone 47) was significantly higher than the sensitivity of FM3.D cells. These data indicate that, in addition to CTL clone 47, clones from groups A and B also could be considered as CTL clones that recognize progression antigens. Recognition of antigen D by the CTL clones 22 and 33 did not change significantly, while lysis of long-term-cultivated FM3 cells by CTL clone 32 was markedly decreased (data not shown).

The sensitivity of FM3 cells to CTL of different recognition groups can be differentially modulated by pretreatment with IFNγ

As noted above, the progressed state of melanoma cells correlates with the level of their expression of MHC class II antigens. IFNγ is a powerful inducer of the expression of MHC class II antigens. It was therefore of interest to investigate any changes in the sensitivity to different CTL clones after pretreatment of melanoma cells with IFNγ. In Fig. 4a the changes in the sensitivity of FM3.D melanoma cells to CTL clones 22 and 47 after treatment with different concentrations of IFNγ are illustrated. The sensitivity to CTL clone 47 was greatly increased, while lysis by CTL clone 22 did not change significantly. The increase in the sensitivity is seen already with low concentrations of IFNγ (1 U/ml). Parallel studies of the changes in the expression

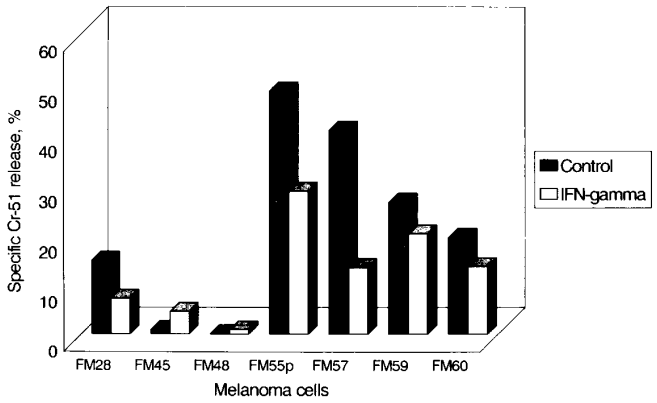

Fig. 5 Modulation of the sensitivity of different target melanoma cell lines to CTL clone 22 by IFNγ treatment. Melanoma cells were treated for 3 days with 25 U/ml IFNγ

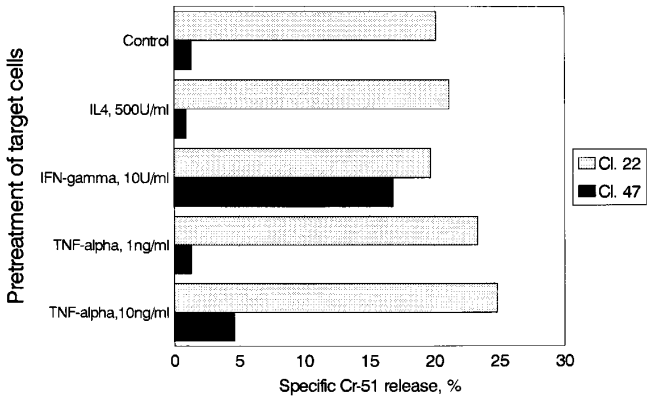

Fig. 6 Comparison of the modulatory effect of pretreatment of melanoma cells with different lymphokines on their sensitivity to CTL clones 22 and 47. Melanoma cells were pretreated for 3 days with either IFNγ (10 U/ml), interleukin-4 (*IL-4*; 500 U/ml) or tumor necrosis factor α (TNF-α; 1 and 10 ng/ml). Percentage specific lysis was determined at a CTL:TC ratio of 10

of MHC and adhesion molecules (Fig. 4b) indicated that only the increase in the level of expression of HLA-DR molecules correlated with the increase in the sensitivity of FM3 cells to CTL clone 47.

It is well known that IFNγ can inhibit the growth of melanoma cells [28, 33]. We investigated the growth-inhibitory activity of IFNγ and found that doses of 1–25 U/ml did not induce significant inhibition of the growth of the majority of melanoma cells tested (data not shown). For all the following experiments we used IFNγ in the concentration range 10–25 U/ml.

While having no significant effect on the recognition of FM3.D cells by CTL clones 22 and 33, treatment the melanoma cell lines FM28, FM55p, FM59 and FM60 with IFNγ induced significant down-regulation of their sensitivity to these CTL clones (Fig. 5). The HLA-A2$^-$ melanoma cell lines FM45 and FM48 were resistant to both clones after treatment with IFNγ (Fig. 5). The cytolytic activity of CTL clone 47 with FM3 cells treated with IFNγ was inhibited by antibodies against monomorphic determinants of MHC class I molecules (W6/32) (Table 2). The HLA-A2-loss variant of $FM55_P$ cells, $FM55_P$.R22, was resistant to clone 47, both before and after treatment with IFNγ (data not shown). All these findings indicate the MHC class I specificity of the observed modulation of sensitivity of melanoma cells to the CTL clones of the different antigen-recognition groups.

Table 2 Action of MHC-class-I-specific mAb (W6/32) on the lytic activity of cytotoxic T lymphocyte (CTL) clone 47 on intact and interferon-γ (IFNγ)-pretreated FM3.D melanoma cells. Melanoma cells were pretreated for 4 days with 25 U/ml IFNγ. ^{51}Cr-labelled melanoma cells were preincubated with 10 μg/ml W6/32 mAb for 1 h in 100 μl, then effector cells were added in 100 μl at a CTL: humor-cell ratio of 10

IFNγ pretreatment	Specific ^{51}Cr release (%) on control cells in the presence of mAb	
–	4.5	1.7
+	41.8	16.8

Comparison of the action of IFNγ with other lymphokines

Other lymphokines have also been shown to be able to modulate the expression of MHC antigens on melanoma cells (e.g. [1]). We first investigated the action of IL-4 and TNFα. The results presented in Fig. 6 indicate no significant changes in the sensitivity of FM3 after treatment with either IL-4 (500 U/ml) or TNFα (10 ng/ml).

IL-10 was selected as a lymphokine having effects contrary to the effects of IFNγ [23]. IL-10 had no effect on the sensitivity of FM3 cells to CTL clones of groups A, B and C in contrast to clones of group D (22 and 32), where the sensitivity of FM3 cells was significantly increased (Fig. 7). Increasing the IL-10 concentration to 100 U/ml did not significantly change this type of modulatory effect (data not shown). Figure 7 also illustrates the increased sensitivity of FM3 cells to CTL clones of groups A and B,

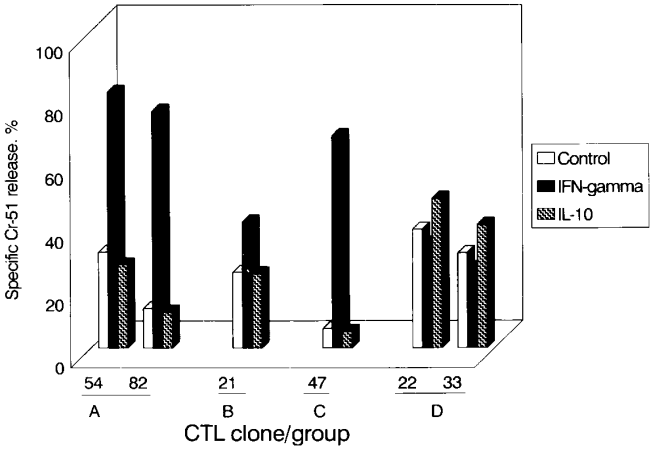

Fig. 7 Modulatory effects of IFNγ and IL-10 on the sensitivity of FM3 cells to CTL clones of different antigen-recognition groups. Melanoma cells were pretreated with either IFNγ (25 U/ml) or IL-10 (25 U/ml). Percentage of specific lysis was determined at a CTL:TC ratio of 10

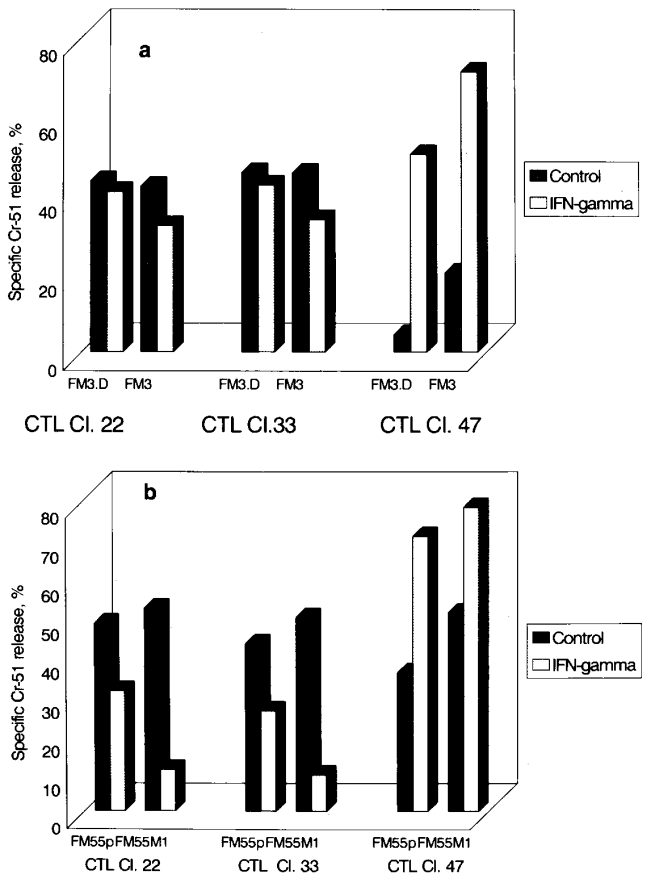

Fig. 8a,b Comparison of the modulatory effect of IFNγ pretreatment of melanoma cells with different states of progression on their sensitivity to CTL clones of group D. **a** FM3 and FM3.D cells; **b** primary and metastatic melanoma cells of patient 55. Melanoma cells were pretreated as in Fig. 6

indicating that the enhanced sensitivity to lysis by CTL clone 47 after treatment of melanoma cells with IFNγ is not a unique property of this particular antigen, but rather reflects a general property of antigens considered as progression antigens.

The down-regulation of the sensitivity of melanoma cells to CTL clones recognizing differentiation antigens is more pronounced with progressed tumor cells

As illustrated in Fig. 5, the down-regulation of the sensitivity of FM55p and FM28 to CTL clone 22 was very pronounced, in comparison to the sensitivity FM3.D cells. These results suggests that the extent of down-regulation could be connected to the differentiation state of melanoma cells, as morphologically FM55p and FM28 appear less differentiated than FM3.D cells. To test this, we compared the modulatory effect of IFNγ on FM3 and FM3.D cells. Both cell lines show a comparable extent of up-regulation of their sensitivity to CTL clone 47, but the sensitivity to CTL clones 22 and 33 was down-regulated in FM3 cells to a higher extent than in FM3.D cells (Fig. 8a). The same

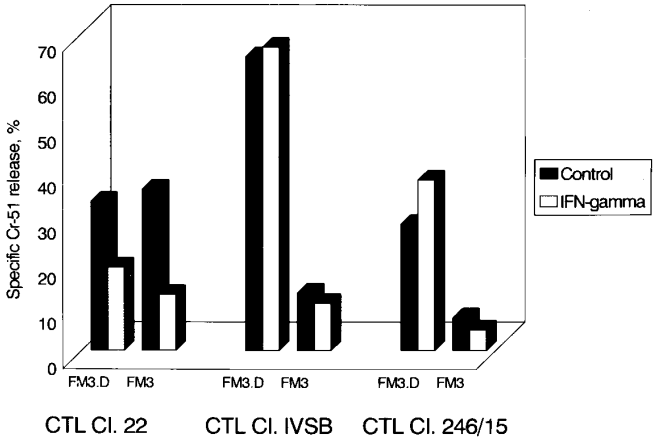

Fig. 9 Comparison of the modulatory effect of the treatment of FM3 and FM3.D melanoma cells with IFNγ on their sensitivity to CTL clone 22 and CTL clones specific for tyrosinase and Melan-A (clones IVSB and 246/15 respectively). Melanoma cells were pretreated with 25 U/ml IFNγ twice for 6 days. Percentsage specific lysis was determined at a CTL:TC ratio of 10

difference in the extent of down-regulation of sensitivity to clones 22 and 33 was noted when primary and metastatic tumor cell lines from patient 55 were compared (Fig. 8b). These findings indicate that the decrease in the sensitivity to CTL clones detecting differentiation antigens is more readily achieved in progressed tumor cells than in differentiated tumor cells. More important, upon repeated treatments of differentiated FM3.D cells with IFNγ, the extent of this down-regulation was greatly increased (compare Figs. 8a and 9).

Treatment of melanoma cells with IFNγ does not significantly modulate the sensitivity of melanoma cells to CTL recognizing known melanoma-associated differentiation antigens

The target antigens for the CTL clones used in the experiments reported above are not known, and therefore their association with differentiation/progression states of melanoma cells are still hypothetical [17]. In order to obtain additional evidence for the differential regulation of differentiation and progression antigens in response to the treatment with IFNγ, we investigated the modulation of the expression of two known markers of differentiation recognized by CTL clones, Melan-A and tyrosinase.

First, we compared the sensitivity of FM3 and FM3.D cells to CTL clones specific for the differentiation antigens, Melan-A (CTL clone 246/15) and tyrosinase (CTL clone IVSB). These results indicate that, unlike CTL clones 22 and 33, the sensitivity of FM3 cells to CTL clones specific for tyrosinase and Melan-A was significantly lower than the sensitivity of FM3.D cells (Fig. 9). Treatment of FM3 and FM3.D cells with IFNγ did not induce significant changes in their sensitivity to lysis, again suggesting the difference between these CTL clones and the CTL clones specific for progression antigens.

Table 3 Comparison of the development of tumors in nude mice after injection of FM3 and FM3.D melanoma cells. Melanoma cells were pretreated for 3 days with 10 U/ml IFNγ, then 5×10^6 melanoma cells were injected subcutaneously in 0.2 ml phosphate-buffered saline and the development of visible tumors was monitored.

Melanoma cells	Pretreatment with IFNγ	Development of visible tumors	
		14 days	28 days
FM3	–	1/5	2/5
	+	4/5	5/5
FM3.D	–	0/5	0/5
	+	0/5	0/5

Quantitative RT-PCR

A quantitative analysis based on RT-PCR was set up in order to investigate differences in the expression of the known melanoma-associated antigens tyrosinase, MART-1/Melan-A, MAGE-3 and gp100 in FM3 and FM3.D. As a control we used the household gene *GAPDH*. The results from duplicate experiments showed no significant differences in the expression of MAGE-3, but the expression levels of tyrosinase, gp100 and MART-1/Melan-A, was significantly lower (35–40%) in FM3 as compared to FM3.D cells.

Treatment of FM3 cells with IFNγ increases their ability to form tumors in nude mice

As shown above, the treatment with IFNγ selectively increases the sensitivity of melanoma cells to CTL specific for progression-associated antigens. This increase could be the result of a general shift in the progression state of melanoma cells. In order to test this, we compared the ability of FM3 and FM3.D cells to form tumors in nude mice with or without pretreatment of tumor cells with IFNγ. The results of one such experiment are summarized in Table 3. The ability to form tumors was much higher for untreated FM3 cells than for untreated FM3.D cells, which corresponds to the data presented above on the more progressed phenotype of FM3 cells. Treatment of FM3 cells with IFNγ significantly increased their ability to form tumors – tumors appeared earlier and in a larger fraction of mice. No stimulation of tumor formation was seen after the treatment of FM3.D cells with IFNγ. These results again indicate that the sensitivity of melanoma cells to the induction of a more progressed state after IFNγ treatment was higher for cells that had already progressed.

Discussion

The main observation of this study is that the sensitivity of melanoma cells to CTL clones recognizing differentiation and progression antigens is differentially modulated during tumor progression and after treatment with the cytokines IFNγ or IL-10. As models for tumor progression we have used two different systems. The first model has been described previously [17] and consists of comparison of primary and metastatic tumor cells, taken from the patient (patient 55) at the same time. It was shown that the sensitivity of metastatic melanoma cells to CTL clones recognizing differentiation antigens was lower, while their sensitivity to CTL clones recognizing progression antigens was higher than for primary melanoma cells. In this study, we have used another model, comparing two different sublines of FM3 cells, differing in their states of differentiation. The original FM3 cell line has been compared with a variant FM3 cell line, FM3.D, which was isolated upon a second attempt to establish a cell line from the original biopsy material. FM3.D has a predominantly dendritic morphology.

These two cell lines also differed in their expression of the melanoma progression marker, HLA-DR (Fig. 2), the higher proliferative rate of FM3 cells (data not shown), the higher content of melanin in FM3.D (data not shown) as well as the lack of tumor formation in nude mice by FM3.D cells (Table 3). Altogether, these data suggest that the FM3.D subline is a differentiated variant of FM3 cells. The differences in the sensitivity of these two FM3 cell lines to the CTL clones from different antigen-recognition groups were very pronounced: FM3 cells showed a significantly higher sensitivity to CTL clones recognizing progression antigens, while the sensitivity to CTL clones recognizing differentiation antigens was either similar (clones 22 and 33) or significantly lower (clone 32, 246/5 and IVSB). In general, such changes were not unexpected, and might be used as additional evidence that these two groups of CTL clones recognize different groups of antigens, i.e. progression and differentiation antigens.

An important question is how the sensitivity to these two groups of CTL clones can be modulated by lymphokines, known to be produced during the interaction of lymphocytes with tumor cells [31]. First we chose to analyze IFNγ. The unexpected finding was that the induced modulation was different for CTL clones recognizing either differentiation or progression antigens. With progression antigens, their sensitivity increased and, in some cases, this was very dramatic. On the other hand, there was either no change in the sensitivity to CTL recognizing differentiation antigens or the sensitivity decreased, depending on the particular CTL clone or melanoma cell line used. The increase in sensitivity to CTL-mediated lysis, also observed by other investigators, was not directly connected to the increase in the expression of MHC class I or adhesion/costimulatory molecules [30, 37]. We also observed that the increase in the expression of MHC class I molecules was insignificant and did not correlate with the increase in sensitivity to lysis by CTL clone 47 (Fig. 5a,b).

Likewise, the increase cannot explain the decrease of the sensitivity to the clones recognizing differentiation antigens (CTL clones 22, 32 and 33). Therefore, these changes are probably associated either with a selective regulation of the expression of differentiation and progression antigens in-

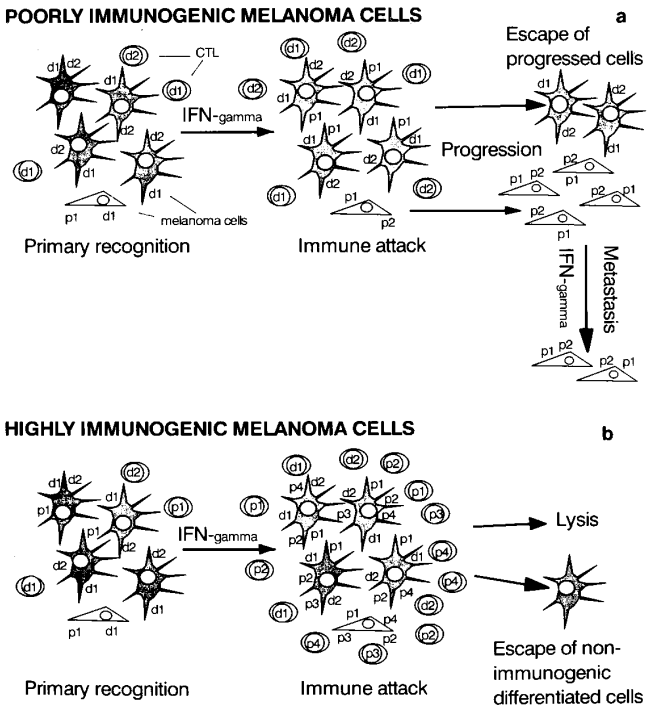

Fig. 10a,b Hypothesis on the possible mechanisms of involvement of the immune response and production of IFNγ in the control of the growth of poorly (**a**) or highly immunogenic (**b**) melanoma cells. Dendritic-like cells are differentiated melanoma cells, while triangular cells are progressed melanoma cells. The antigens designated d1, d2 and p1−p4 on the surface of the melanoma cells represent different differentiation and progression antigens respectively. The same letters inside the schematically drawn lymphocytes represent the antigen specificity of these lymphocytes

duced by IFNγ pretreatment, or with a selective change in the association of these two groups of antigens to the MHC class I molecules inside the cells. At present, we cannot discriminate between these two possibilities for antigens recognized by the CTL clones described, but it is of interest to note that we did not observe significant changes in the level of mRNA for tyrosinase and Melan-A measured by RT-PCR after treatment with IFNγ, and also that no increase in the sensitivity to lysis for CTL clones recognizing these antigens was observed (Fig. 9). On the other hand, the decreased sensitivity of FM3 cells in comparison with FM3.D cells to CTL clones specific for tyrosinase and Melan-A correlated with decreased levels of the expression of mRNA for these antigens in FM3 cells.

These data suggest the possibility that the efficiency of the presentation of these antigens to CTL was not changed after treatment with IFNγ. Alternatively, the increased sensitivity of melanoma cells after treatment with IFNγ to CTL clones recognizing progression antigens could be associated with an increase in the expression of their genes. It is of interest that HLA class II antigens and ICAM-1, the expression of which are known to be increased in melanoma cells after IFNγ treatment, are in fact expressed like progression-associated antigens. The increase in the expression of other progression-associated antigens after IFNγ treatment has been noted before by others [18].

Of special interest is the differential sensitivity of melanoma cells to down-regulation of recognition by the CTL clones 22, 32 and 33 by treatment with IFNγ. This was shown both for melanoma cells of primary and metastatic origin from patient 55 (Fig. 8b) and for FM3.D and FM3 melanoma cells (Fig. 8a). The progressed tumor cells were more sensitive to this type of down-regulation, which could be explained by a less stable differentiated state of cells after their progression. This difference was not equally evident with CTL clones recognizing the antigens tyrosinase and Melan-A (Fig. 9), but for these clones the recognition of tumor cells was significantly decreased during tumor progression. It was shown, that the sensitivity of human melanoma cells to natural-killer(NK)- and lymphokine-activated-killer(LAK)-cell-mediated lysis decreased after treatment with IFNγ [40], and therefore the possibility cannot be excluded that the down-regulation of the sensitivity of melanoma cells to the lysis by CTL clones of group D could reflect their NK-like behaviour. However, this possibility seems unlikely because (a) the activity of the CTL of this group was significantly inhibited by the pretreatment of melanoma cells with HLA-class I- or HLA-A2-specific monoclonal antibodies [17], and (b) we did not observe any ability of these CTL clones to lyse Daudi or K562 cells (data not shown). In all experiments with CTL of groups A, B and C, recognizing progression antigens, their activity was significantly blocked by the pretreatment of target cells with HLA-class-I-specific antibodies (Table 2, and data not shown), thereby ruling out the possibility of a significant unspecific activity.

The results reported in this paper have implications for the way in which the immune response may influence the progression of melanoma cells in the host organism. This is schematically illustrated in Fig. 10. The hypothesis presented is based on the assumption that melanoma cells may exhibit different levels of immunogenicity which may be dependent on the level of expression of strong tumor-specific antigens [17]. For poorly immunogenic tumor cells (Fig. 10a), the initial immune response is initiated by the presence of the differentiation antigens. Recognition of such tumor cells by $CD8^+$ T cells leads to the lysis of some of these tumor cells with concomitant production of IFNγ [31]. IFNγ can also be produced by a subpopulation of T-helper cells as well as NK cells [24, 32]. IFNγ can induce down-regulation of the sensitivity of melanoma cells to these CTL which, for some differentiation antigens, would be more profound for cells having a more progressed phenotype. This down-regulation would cause the selective escape of progressed cells from the lysis by CTL specific for these differentiation antigens. For other differentiation antigens such as tyrosinase, the progressed state is already associated with a decreased sensitivity to the specific CTL. IFNγ production also could increase the expression of tumor-associated progression antigens but, either because of their low level of expression in poorly immunogenic tumors or a low level of reaction to IFNγ, the generation of CTL specific for these antigens is inefficient and the tumor

continues to grow. As a result, the immune response could in fact support tumor progression. It is important to note that IFNγ may have this direct tumor-promoting effect on melanoma cells, as it has been shown that, following treatment with IFNγ, melanoma cells acquire a more malignant phenotype [9] and even have shown increased metastatic ability [19, 21].

Further preliminary data also indicate that the ability of FM3 cells, but not FM3.D cells, to form tumors in nude mice was also increased after such treatment (Table 3). However, it should be noted that the exact mechanism of the increased ability of IFNγ-treated FM3 cells to form tumors in nude mice is not known and could be mediated either by increased proliferative ability of melanoma cells or by decreased sensitivity to NK-mediated lysis. The experiments to test these possibilities are now in progress.

In the case of highly immunogenic tumors (Fig. 10b), the initial recognition of differentiation antigens and possibly some of the progression antigens may lead to a significant increase in the expression of progression antigens. This, in turn, can lead to the development of the immune response and the formation of effector CTL against these progression antigens and, as a result, induce the regression of tumor.

A central element of this hypothesis is that some lymphokines such as IFNγ may play different roles during the immune response against melanoma cells of different degrees of immunogenicity, increasing the efficiency of the immune response against highly immunogenic tumor cells and increasing the possibility of tumor progression in the case of poorly immunogenic tumor cells. IL-10 possibly can play the opposite role, decreasing the response against highly immunogenic tumor cells and increasing the response against poorly immunogenic tumor cells. It is relevant that we could not detect significant down-regulation of the sensitivity of melanoma cells to CTL clones using IL-10, which has also been observed by others [20]. This discrepancy could be the result of the differences in the experimental systems (for example, we pretreated melanoma cells with IL-10 in serum-containing medium) or low sensitivity of the particular cell line (FM3) to the down-regulation by IL-10, and experiments are now in progress to analyze these differences.

IFNγ is also known to be able to increase the activity of NK cells [32] and has been suggested for use in clinical trials with melanoma patients, but the clinical results were not promising [8, 13] and some trials were interrupted apparently because of stimulation of the processes of tumor progression [22]. The data presented in this paper provide some possible explanations for the unsuspected adverse effects of IFNγ in clinical trials, as well as for the immunostimulatory role of lymphocyte populations in tumor growth [26, 38], which should be a subject for future studies.

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