

The decreased susceptibility of metastatic melanoma cells to killing involves an alteration of CTL reactivity

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Abstract. Metastases are known to be more resistant to therapy than matching primary tumors, in particular they are less prone to apoptosis. In this study we investigated the functional interaction of a CTL clone (LT12) specific for a melanoma TA with the primary tumor (T1) versus its metastatic counterpart (G1). The CTL clone (LT12) was shown to lyse the primary T1 cells more efficiently in a classical cytotoxicity test. This differential susceptibility was not associated with MHC class I down-regulation and conjugate formation but correlated with a differential increase in Ca⁺⁺ flux in the LT12 CTL when stimulated with the primary versus the metastatic tumor cells. Since LT12 uses perforin/granzyme B to kill its autologous target we analysed perforin and granzyme B mRNA expression in the CTL in the presence of either primary and metastatic melanoma cells. Quantitative PCR analysis showed an increased expression of granzyme B and perforin mRNA levels in LT12 when cocultured in the presence of the primary tumor. However, a similar level of (cytotoxic molecule) degranulation as revealed by CD107 expression was observed when LT12 was stimulated with T1 or G1 cells. These data suggest that the differential susceptibility of primary and metastatic melanoma cells involves at least in part their distinct potential to induce autologous CTL reactivity and the subsequent triggering of granzyme B and perforin in these cells.

Introduction

Melanoma is the tumor with the fastest growing incidence among Caucasians. While early detection and surgery of primary melanoma improves the outcome, metastatic melanoma is still largely refractory to therapy (1). As melanoma progresses to metastatic disease, powerful mechanisms of tumor evasion from immune recognition are activated thus tilting the balance between immunity and escape in favor of tumor resistance to host defense. Thanks to recent progress in tumor immunology, immunotherapy trials have been carried out; however, very little success has been achieved so far (2). At the present time, the molecular pathogenesis of melanoma is still largely unknown and both classical and biological tumor therapy approaches to cure metastatic melanoma are largely inefficient. In this regard, expression profiling has identified clusters and identified genes of interest that could be targeted in future therapies (3). Furthermore, response to IL-2 therapy defines clusters of response, as with other sets of data, no correlation with clinical outcome was established for melanoma patients (3,4).

The most effective immunologically mediated host strategy is the deployment of cytotoxic T lymphocytes that can directly lyse targets and have the capacity to secrete effector cytokines such as interferon γ and tumor necrosis factor α (5). In the T cell-mediated cytotoxicity process, two major pathways are engaged following TCR recognition of Ag/MHC complexes expressed on target cells. The first one is a secretory pathway involving receptor-triggered exocytosis of performed secretory granules containing granzymes and perforin (6,7). The second is based on receptor-induced surface expression of death receptor ligands on effector cells, which cross-links the corresponding receptors (Fas, TRAIL receptors, TNFR I-p55) on target cells (8). In mice and humans, granzymes A and B are the most abundant granzymes and have received the most attention, in particular granzyme B. Several lines of evidence indicate that tumor cells proliferate under adverse host conditions during tumor progression, using several strategies to adapt their survival by blocking the action of key regulators of the immune response and circumventing anti-tumor defenses. Despite the expression of tumor-associated antigens, tumor eradication by the immune system is often inefficient (9). In

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Abbreviations: mAb, monoclonal antibody; CTL, cytotoxic T lymphocyte; MICA, MHC class I-related chain A; MICB, MHC class I-related chain B

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this regard, it has been reported that metastatic cancer cells exhibit a higher resistance to apoptosis as compared to their poorly-metastatic counterparts, which indicates that metastatic cancer cells may have more dysregulations in apoptosis pathways (10-12). However, there are only few experimental data linking tumor susceptibility to CTL-induced cell death and metastasis (13-16). It is well established that the outcome of the antitumor response is the result of cross-talk between effector cells and the tumor target submitted to the influence of the tumor microenvironment. Therefore, the present study was undertaken to investigate the primary versus metastatic tumor susceptibility to CTL-mediated killing and their reciprocal functional interaction with these effectors. For this purpose, we used a previously described system comprising a CTL clone, LT12, and the autologous primary T1 and metastatic G1 melanoma cell lines (17). We show that besides the differential susceptibility to lysis, primary and metastatic targets induce a differential reactivity of the autologous CTL clone.

Materials and methods

Cells. Both primary tumor and lymph node metastases were obtained in 1995 from patient Fon, a 77-year-old male with a primary facial melanoma lesion. Tumor samples were mechanically dissociated and aliquots of the resulting single cell suspension maintained in culture for both tumor-infiltrating lymphocyte (TIL) expansion and tumor cell line generation. The LT 12 CTL clone was isolated from autologous TIL as described previously (12). T1 Fon and G1 Fon melanoma cell lines were derived from the primary lesion (T1) and from an invaded lymph node (G1).

Cytolytic activity assay. Standard 4-h ^{51}Cr release assays were used to assess antigen-specific target cell lysis. Target cells (10^6) were labelled with $100 \mu\text{Ci Na}_2^{51}\text{CrO}_4$ (Perkin-Elmer Life Sciences, St. Laurent, Quebec) for 1 h at 37°C , washed three times with RPMI media and then diluted to 2×10^4 cells/ml in complete media. In triplicate, $100 \mu\text{l}$ of target cells were seeded into 96-well V-bottom Librio/Titertek tissue culture plates (ICN Biomedicals, Aurora, OH) with $100 \mu\text{l}$ effector cells (to give effector:target ratios of 30:1, 10:1, 5:1 and 1:1) and cultures were then incubated for 4 h at 37°C . Released ^{51}Cr from supernatants was measured on a Wallac 1470 γ -counter (Wallac Oy, Turku, Finland). Results were expressed in lytic units (LU). LU were calculated as described by Pross *et al.* (18). One LU was defined as the number of effector cells required for 30% lysis of 3×10^3 target cells and the number of LU present in 10^6 effector cells was calculated using a computer program. Inhibition of the granzyme mediated cytotoxic pathway was performed by preincubation of effector cells for 2 h with 100 nM concanamycin A (CMA) (Sigma, France). Target cells were then added to the wells with CMA-treated effector cells (concentration of CMA during lysis: 50 nM).

Antibodies. T1 and G1 cells were stained with a panel of monoclonal antibodies (mAb) which included anti-HLA class I (19), anti-MICA (M673, Amgen), and anti-MICB (M364, Amgen). mAb were used to stain cells in an indirect immunofluorescence. Following incubation, the cells were washed

and analysed in a FACScalibur™ (BD Bioscience, Le Pont de Claix, France).

Measurement of TNF β production by CTLs. LT12 cells were incubated with T1 or G1 tumor cells at a lymphocyte:tumor ratio of 1:6. The supernatants were collected after 12 h, and the TNF β content was detected by measuring the cytotoxicity of the culture medium on the TNF-sensitive WEHI-164c13 cells, with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide colorimetric assay (20).

Analysis of raft distribution by confocal microscopy. As previously described (21), T cells were incubated with $8 \mu\text{g/ml}$ of cholera toxin Alexa Fluor conjugate (Molecular Probes) for 35 min in ice-cold PBS. After washing, CT-loaded T cells were dropped onto target cells previously attached to a poly-L-lysine-coated slide. Confocal microscopy LSM510 (Zeiss, Hallbergmoos, Germany) was performed at 5, 30, 60 and 120 min of coculture.

Videomicroscopy and intracellular calcium concentration assessment. T cells were labelled with $3 \mu\text{M}$ of Fluo4-AM (Molecular Probes, OR) for 15 min at 37°C , washed twice and incubated in RPMI-1640 + 10% FCS for 30 min before coculture with target cells on a glass slide. Video microscopy was performed for 1 h, in HEPES-buffered medium in a temperature-controlled chamber. Pictures were acquired every 10 sec. An increase in fluorescence intensity of at least 50% above the background level was considered significant for calcium influx.

CD107a lysosome-associated membrane protein (LAMP-1) expression assay. Degranulation of intracellular vesicles by lymphocytes can be measured using CD107a, as described recently for CD8 $^+$ T cells (22). Tumoral cells and CD8 $^+$ effector cells, were mixed in a 1:2 target:effector ratio in the presence of $1 \mu\text{g/ml}$ CD107a-PeCy5 Ab (BD Biosciences) for 30, 60, 90 or 180 min at 37°C . Samples were then surface-stained using CD3-FITC (BD Biosciences) for 30 min, washed and fixed with 3.7% paraformaldehyde (Sigma-Aldrich). Flow cytometry analysis for cell surface CD107a expression was performed on gated lymphocytes.

Real-time quantitative reverse transcription-PCR analysis. For analysis of granzyme B and perforin induction, LT12 was conjugated with T1 or G1 cells at a 2:1 ratio for 2 and 3 h. RNA extraction was performed with TRIzol and reverse transcribed. PCR primers and probes for the granzyme B and perforin genes were designed by Applied Biosystems and used according to the manufacturer's recommendations. The amount of sample RNA was normalized by the amplification of an endogenous control (18S). The relative quantification of the transcripts was derived using the standard curve method (Applied Biosystems User Bulletin 2, ABI PRISM 7700 Sequence Detection system).

Results

Differential susceptibility of primary T1 and metastatic G1 melanoma cells to killing by autologous CTL clone. We first

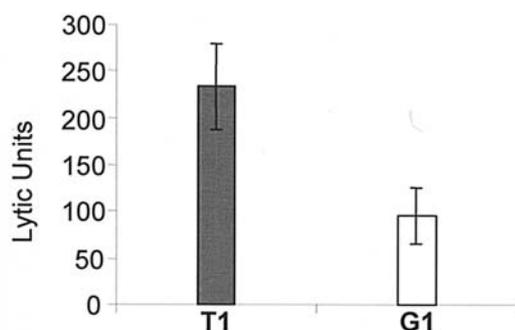


Figure 1. LT12 mediated killing of primary (T1) and metastatic (G1) melanoma cells. Chromium (^{51}Cr) labelled melanoma cells were incubated at 37°C with LT12 CTL cells at different ratios. After 4 h, the supernatants were collected and analysed in a γ -counter. Lysis was expressed as lytic units (LU-30). One lytic unit is defined as the number of LT12 cells required for 30% lysis of 3×10^3 target cells and the number of LU present in 10^6 effector cells is presented.

investigated the susceptibility of the primary melanoma cell line, T1, and its metastatic derivative, G1, to the cytotoxicity induced by the autologous CTL clone, LT12, using the standard chromium release assay. As shown in Fig. 1, while primitive melanoma cells were significantly killed (234 LU) by the CTL clone, a significant decrease in susceptibility to lysis (96 LU) was observed when G1 metastatic melanoma cells were used as targets.

The differential susceptibility of primary and metastatic targets is not due to down-regulation of MHC class I molecule expression. The loss of MHC class I by metastatic cells has been previously reported (23,24). In order to examine whether a relationship exists between expression of MHC class I and decreased susceptibility to CTL killing, we investigated the expression of MHC class I, MICA and MICB on T1 and G1 cells. Using flow cytometry analysis, we demonstrated a similar surface expression of MHC class I molecules on T1 and G1 cells as revealed by cell staining with W6/32 antibody (Fig. 2A). Because LT12 expresses a high level of NKG2D (data not shown), we also examined its ligand expression by primary and metastatic tumor cells. The data depicted in this figure show that the expression of MICA and MICB by T1 and G1 cells was similarly low and suggest that the observed resistance of metastatic cells to CTL lysis is independent of class I MHC expression.

Furthermore, TNF secretion by LT12 was evaluated in order to examine target recognition by the LT12 CTL clone. As depicted in Fig. 2B, a similar amount of TNF was secreted by the CTL clone stimulated with T1 or G1 cells, ruling out impaired cellular antigen processing and/or presentation.

Conjugate formation between the CTL and primary or metastatic tumor cells. Accumulated actin at the contact between T and antigen presenting cells (APCs) was found to stabilize a continuous contact that allows the clustering of TCRs and costimulatory signaling molecules into lipid rafts (25). As shown in Fig. 3, using confocal microscopy, we observed rapid conjugate formation (at 5 min) between the LT12 CTL clone and T1 or G1 tumor cells. This figure shows a tight polarization of the fluorescence at contact site with similar lipid

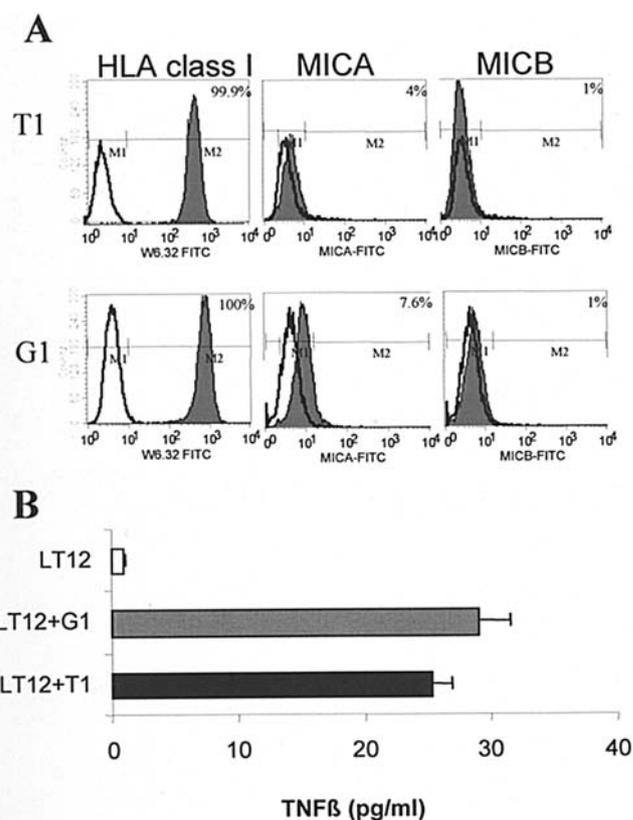


Figure 2. (A) Cell surface expression of MHC class I, MICA and MICB on target cells. T1 and G1 were stained with FITC-conjugated anti-MHC class I, anti-MICA and anti-MICB. Black-shaded histograms are for positive cells and white-shaded histogram for mouse isotype control (IgG). Values indicate the percentage of positive cells. (B) TNF β production by cytotoxic T lymphocytes in response to the primary and the metastatic cells. After 12 h of coculture of CTL and tumor cells, the supernatants were analysed by an MTT test in triplicate.

raft coalescence in the presence of both primary and metastatic cells. This suggests that the decreased susceptibility of metastatic melanoma cells to killing is not the likely consequence of an alteration of aggregation of lipid rafts and thus not attributable to a defect in cellular interaction between effector CTL and target cells.

Differential calcium flux in autologous CTL in the presence of primitive and metastatic melanoma. Having shown the differential susceptibility of primary versus metastatic cells to CTL-mediated lysis, we then analysed the Ca^{2+} response in LT12 CTL as a measure for early T cell recognition and reactivation events. LT12 CTL were labelled with Fluo4-AM before incubation in the presence of either T1 or G1 melanoma cell lines. The Ca^{2+} response was assessed using video microscopy. As shown in Fig. 4, increased calcium influx was observed in LT12 CTL when cocultured with the primary T1 cells. However, when G1 metastatic cells were used for stimulation, only a marginal increase in Ca^{2+} flux was observed in the LT12 CTL clone.

LT12 uses the perforin/granzyme pathway to kill T1 and G1 targets. T1 and G1 cells were used as targets of CTL in the presence or absence of CMA (to chelate Ca^{2+}). As shown in Fig. 5A, an inhibition of exocytosis mediated pathway by

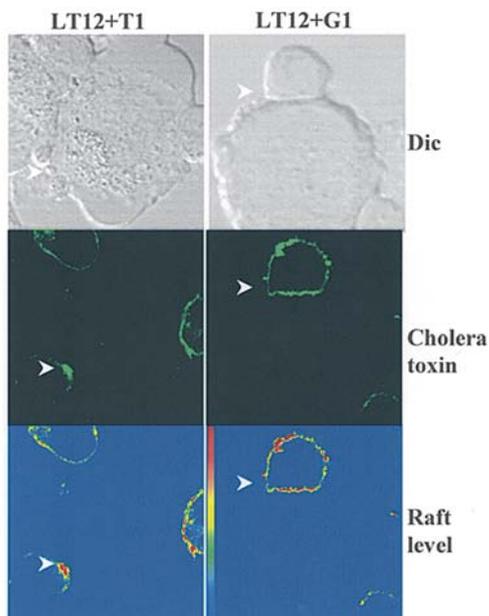


Figure 3. Immunological synapse formation between LT12 CTL and the primary or metastatic cells. After 5, 30, 60 and 120 min of coculture at 37°C, the cells were fixed with 4% paraformaldehyde and mounted on Vectashield. Conjugates were examined using confocal microscopy. Synapse formation at 5 min of coculture is shown. Data are from one representative experiment of three.

concomycin A resulted in the abrogation of LT12 CTL clone cytotoxicity, indicating that the observed cytotoxicity towards both T1 and G1 cells is mediated by the perforin/granzymes pathway. Further, we examined the degranulation ability of CTLs in the presence of primary or metastatic cells. As illustrated in Fig. 5B, the percentage of degranulation was similar at different times following LT12 conjugation with T1 targets or G1 targets.

Down-regulation of perforin, granzyme B mRNA expression in LT12 CTL stimulated by metastatic tumor cells. We have shown that the LT12 CTL clone mainly used the secretory pathway to lyse T1 and G1 targets. To investigate the stimulatory potential of T1 primary and G1 metastatic tumor cells, LT12 CTL clone was incubated in the presence of either T1 or G1 cells. Quantitative PCR analysis demonstrated that stimulation of the CTL clone by T1 cells resulted in significant granzyme B transcription while no change in this gene transcription was observed when G1 cells were used as stimulatory cells (Fig. 6). Fig. 6 also shows that, in LT12 cells stimulated either with T1 or G1 cells, perforin gene transcription was very low when LT12 was stimulated with G1 cells. Such transcription even decreased following stimulation with both cell types, confirming that granzyme B and perforin are not under the same transcriptional control in activated CTLs.

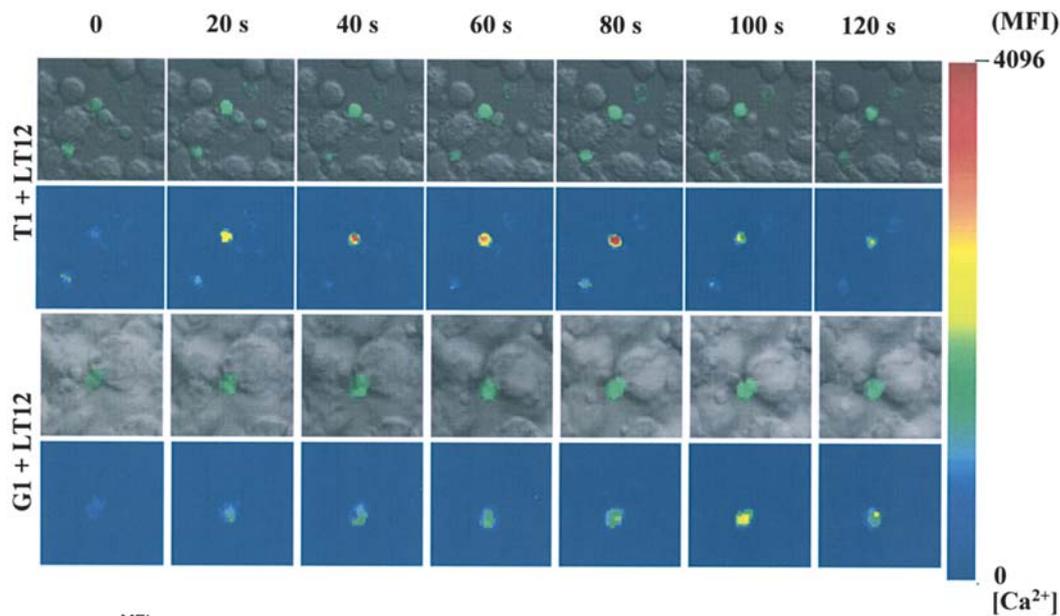


Figure 4. Amplitude of Ca²⁺ response of LT12 CTL to primary or metastatic melanoma cells. The Ca²⁺ response of LT12 cells to T1 and G1 cells was followed during conjugate formation in individual cells during a 1-h time course. The representative pictures of conjugate formation between T1 cells or G1 cells and LT12 are shown. An increase in the intensity of Fluo-4 fluorescence of at least 50% above the level was considered significant. The fluorescence intensity scale is shown on a gray level ranging from 0 to 4096. The data reported are representative of three independent experiments.

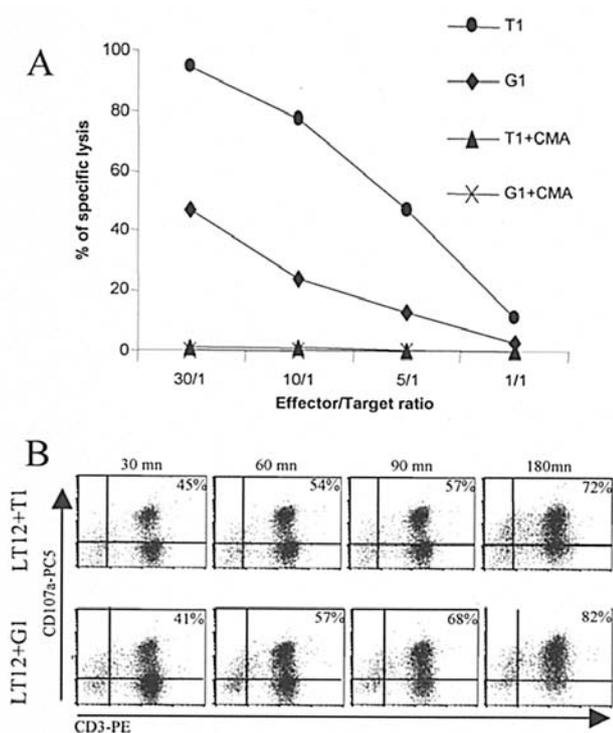


Figure 5. (A) CMA blocks LT12 CTL killing of T1 and G1 cells. Cytotoxicity was determined using a 4-h ^{51}Cr release assay in the presence or absence of Ca^{2+} chelator CMA. (B) CD107a expression assay. LT12 CTLs were stained with PC5-conjugated anti-CD107a and PE-conjugated anti-CD3 after 30, 60, 90 and 180 min of coculture with T1 and G1 cells. Values indicate percentage of positive cells.

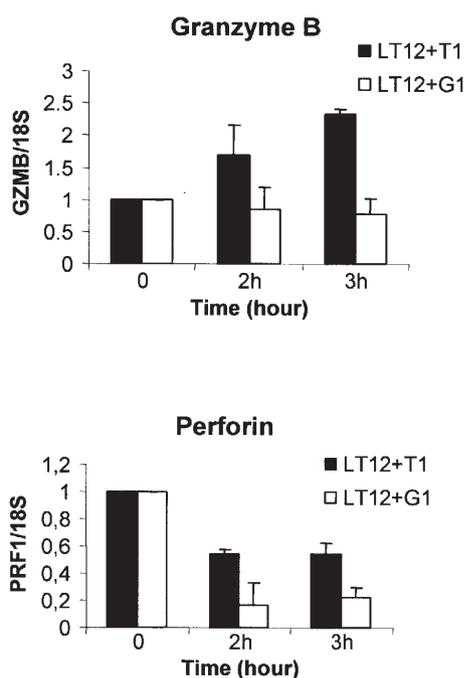


Figure 6. mRNA expression of perforin, granzyme B in LT12 CTL. LT12 was cocultured with T1 or G1 cells for 120 and 180 min. At the end of coculture, cells were collected and the total RNA was extracted and followed by quantitative PCR analysis for granzyme B and perforin gene expression. The amount of sample RNA was normalized by the amplification of an endogenous control (18S). The mixture of LT12 with T1 or G1 cells without incubation (represented by a time of 0 min) was used for calibration. The data reported are representative of two independent experiments.

Discussion

In human melanoma, early detection and excision of primary tumors has improved prognosis but treatment of metastases is still very inefficient with 5-year survival rates of 6% (26). Although, metastatic cells were previously found to be more resistant to apoptosis induced by cytotoxic treatments (12), little is known about the relative susceptibility of metastatic tumor cells to CTL-mediated lysis. Thanks to important progress made over the past ten years, biotherapy and, in particular, targeted immunotherapy protocols could be developed. However, despite the ability to induce tumor antigen-specific cytotoxic T cells, success has been very rare (27,28). It is known that malignant transformation is associated with genetic alterations providing tumor cells with mechanisms for escape from immune surveillance. The data of the present studies point to a decreased susceptibility to CTL-induced lysis of metastatic melanoma cells as compared to that of their primary equivalents. These results are in agreement with the report of Thompson *et al* that both primary and metastatic prostate cells induced antitumor CTLs in syngeneic hosts but, unlike the primary cells, the metastatic cells were resistant to CTL lysis (29). Furthermore, the existence of a dominant factor in metastatic prostate cancer cells that is able to confer specific protection against CTL cytotoxicity has been suggested. One of the mechanisms for the immune escape phenotype in tumor cells is loss or reduction of class I MHC expression (30). In this regard, Takenoyama *et al* have reported that, although HLA class I was maintained in a primary and metastatic human lung carcinoma established from one patient, the CTL clone lysed only the primary cells (31). These results agree with our finding that, however similar the level of class I MHC molecule expression was, a significant decrease in metastatic melanoma susceptibility to killing by CTLs occurred. This suggests that, during tumor progression, metastatic cancer cells may acquire various complex mechanisms to evade cellular destruction by CTLs.

Since it has been reported that NKG2D serves as a co-stimulatory receptor for TCR-mediated signals in a manner similar to CD28 (32,33) and that a low expression of its ligands, MICA and MICB, was responsible for the resistance of some tumor cells to lysis by effector cells (34), we asked whether an altered expression of this receptor or its ligands on metastatic cells could explain the differential susceptibility to specific cell-mediated cytotoxicity. Our data clearly indicate that primary and metastatic tumor cells express similar low levels of MICA and MICB, suggesting that the expression of NKG2D ligands by metastatic tumor cells is not involved in the down-regulated CTL reactivity induced by metastatic cells. When TNF secretion by CTLs cocultured with primary and metastatic cells was measured, the same level was observed. This suggests that a loss of recognition of the metastatic tumor is probably not involved in G1 resistance to CTL-mediated killing.

Despite the similar conjugate formation between CTL and primary or metastatic cells, we noticed a differential Ca^{2+} flux in the LT12 CTL clone following its interaction with T1 or G1 tumor cells. Obviously, a more pronounced increase in calcium influx was observed in the presence of the primary cells. As calcium influx has been shown to play an important

role in the induction of CTL activity (25), it is tempting to speculate that the differential susceptibility of primary and metastatic melanoma to killing by CTL can be explained, at least in part, by a more efficient functional CTL interaction with the primary versus the metastatic clone. Such interaction would be important to trigger the lytic potential of CTLs. We observed a stronger expression of perforin and granzyme B mRNA in the CTLs when cocultured in the presence of the primary cells as compared to metastatic tumors, suggesting that primary cells have a more pronounced stimulatory potential to trigger cytotoxic molecule transcription in effector cells. Nevertheless, monitoring the presence or absence of perforin and granzyme gives no indication of the ability of T cells to degranulate. So, we examined the degranulation of the CTL clone (LT12) at different times of coculture with T1 and G1. We demonstrated that CTLs displayed the same degranulation capacity in the presence of primary and metastatic cells. This suggests the absence of an inhibitory factor of degranulation in CTLs in the presence of metastatic cells which was considered one of the mechanisms of immune escape (35).

Overall, our data indicate that the differential response to CTL-mediated cytolysis may be related to a difference in cytotoxic molecule transcription following CTL recognition of primary and metastatic tumor cells. Immune response induced by immunotherapy may eliminate a majority of primary cancer cells but a proportion of metastatic cancer cells could remain resistant to immune attack. Therefore, to optimise immunotherapy strategies, correction of immune activating signals, eradication of inhibitory factors and evasion from newly developed immunoresistant tumor phenotypes need to be simultaneously considered.

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