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ORIGINAL ARTICLE

Tissue transglutaminase activity protects from cutaneous melanoma metastatic dissemination: an in vivo study

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Abstract The role of tissue transglutaminase (TG-2, TGase-2) in cancer development is still a fascinating field of research. The available reports do not elucidate fully its mechanism of action, due to the limitations of in vitro approaches. Therefore, to understand TG-2 role in cancer, we carried out an in vivo study with a more direct approach. TG-2 was in vivo overexpressed in a murine model of melanoma (intravenous injection of B16 melanoma cells in C57BL/6N mice) by means of a plasmid carrying the TG-2 cDNA. The evaluation of the frequency and size of the metastases indicated that the number of melanoma lung foci was more markedly reduced by TG-2 overexpression than the metastatic size. Then, TG-2 overexpressing mice showed a prolonged survival with respect to control mice. Further analyses were carried by means of proteomic analysis of melanoma cell lysates and meta-analysis of published transcriptomic datasets. Proteomic analysis of cell lysates from a human melanoma cell line compared to human keratinocytes showed significant differences in the expression of TG-2 substrates known to be involved in proliferation/differentiation and cancer progression. Taken together, these findings indicate a protective role of TG-2 enzymatic activity in melanoma progression in vivo.

Keywords Cutaneous melanoma · Transglutaminase type 2 · Microenvironment · Cell proteome · Bioinformatics

Abbreviations

ASC	Active site collection
BAEC	Bovine aortic endothelial cell
CID	Collision-induced dissociation
DME	Dulbecco's modified Eagle medium
ECM	Extra-cellular matrix
GI	Growth index
II	Invasion index
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer solution
RP-LC–MS/MS	Reversed-phase liquid chromatography tandem mass spectrometry
SDS	Sodium dodecylsulfate
TGase or TG	Transglutaminase
TRANSIT	TRANSglutaminase SITes

Introduction

Cutaneous melanoma is a cutaneous cancer developing from melanocytes, i.e. melanin pigment producing cells. Primary melanoma usually starts from skin, less frequently

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from other pigmented tissues, such as the eye, the bowel or others. Among human cancers, it is considered one of the most aggressive and with the highest mortality rate (Jemal et al. 2009). Valid therapeutic options for the advanced melanoma phases are still lacking. In fact, cutaneous melanoma can be effectively treated by surgical excision only at early stages; therefore, the prompt detection, diagnosis, and adequate removal of such lesions are of enormous importance. On the other hand, the knowledge about the molecular mechanisms involved in the metastatic dissemination is poor. Therefore, to better understand melanoma cells biology, there is a large interest to develop new effective drugs which might be useful to influence future therapeutic approaches (Sosman and Puzanov 2006; Markovic et al. 2007). Recent scientific investigations have highlighted genetic alterations and biological processes that seem to be critical to melanoma cell survival and resistance to standard therapy, e.g. BRAF, S100, amyloid and others (Maurer et al. 2011; Blank et al. 2011; Hong et al. 2011).

The involvement of tissue transglutaminase (TG-2) in apoptotic processes as well as in cell growth and differentiation (Knight et al. 1991; Fesus 1993; Fesus and Piacentini 2002; Griffin et al. 2002) strongly suggests a role in cancer growth and development, although its relevance is not yet well understood. TG-2 is a multifunctional enzyme, which may act as a transamidating enzyme able to cross-link proteins, or covalently modify them, in programmed cell death (Fesus and Piacentini 2002) as a G-protein, in adrenoreceptor signaling, as a protein disulphide isomerase, in maintaining mitochondrial physiology, or as secreted protein, in the stabilization of the extra-cellular matrix (ECM). Further, TG-2 was also reported to possess glutamine deaminase and kinase enzymatic activities, suggesting a complex and intriguing role of TG-2 in maintaining protein folding, polyamine and nucleotide content intra- and extracellularly (Facchiano and Facchiano 2009). In fact, TGs can catalyze the covalent incorporation of several low molecular weight amines into proteins, in the form of amides of the γ-carboxyl group of a peptide-bound glutamic acid (Mycek et al. 1959). Moreover, this reaction was reported to occur even with biogenic amines like serotonin, adrenaline or histamine (Walther et al. 2003). TG-catalyzed reactions are specific for a particular glutamine residue in native protein substrates (Greenberg et al. 1991). The role of TG-2 in the post-translational modification of ECM and basement membrane proteins in the metastatic process was extensively investigated in B16-F10 melanoma cells (Lentini et al. 2008). In particular, it was observed that cancer cells exhibit a lower TG-2 transamidating activity than their normal counterparts (Beninati et al. 1993; Beninati 1995). It is also noteworthy that the induction of TG-2 activity by powerful natural agents, such as retinoids and methylxanthines, leads to an effective switch to cell differentiation and apoptotic death. The decline of TG-2 transamidating activity in tumors is potentially a bad prognostic biomarker and is possibly related to tumor metastatic potential, by determining the ability of tumor cells to cross basement membranes and to invade the bloodstream. Given the different proposed functions of TG-2, a reduced enzyme expression and transamidating activity in tumors would indeed lead to reduced cell adhesion, increased migration and a less stable ECM, thus facilitating the first step of the invasive process by cancer cells (Tabolacci et al. 2012). Furthermore, it has been well established that the highly metastatic B16-F10 murine melanoma cell line do not express TG-2 at detectable levels, and the number of metastases developed in melanoma-bearing TG-$2^{-/-}$ mice is higher than that developed in wild type ones (Di Giacomo et al. 2009). It is noteworthy that while a number of reported studies suggested a possible anti-tumor role of TG-2 in melanoma cancer proliferation and invasion, likely by inducing cell apoptosis or promoting differentiation of melanoma cells (Bergamini et al. 2003; Lentini et al. 2009; Di Giacomo et al. 2009; Gismondi et al. 2010), other studies suggested an opposite role (Chhabra et al. 2009; Mehta et al. 2010). These discrepancies might be, at least partially, explained by the differences between the in vitro and in vivo models used, and by the difficulty to apply general conclusions deriving from cellular models to more complex animal or human disease.

Therefore, in the attempt to clarify this controversial role of TG-2, the aim of the present study was to investigate more directly the role of its transamidating enzymatic activity on cutaneous melanoma aggressiveness, by inducing its overexpression in mice, measuring the increased transamidating enzymatic activity and comparing the effects. Further, a preliminary direct evaluation of TG-2 expression on human cutaneous melanoma specimens from patients, compared to healthy controls, was carried out.

Materials and methods

Cell culture

Highly metastatic B16-F10 murine melanoma cells (obtained from ATCC, Manassas,VA) were propagated under standard culture conditions. Cultures were found to be free from Mycoplasma species using the Hoechst staining procedure. The invasive potential was assessed in an in vitro invasion assay as previously described (Albini et al. 1987). The metastatic activity, both spontaneous and experimental, was found to be stable over the time of the experimental procedures. Cell viability was tested by the

Trypan Blue (0.25 %) exclusion test at different times of culturing. Cells were washed twice with DMEM, harvested with trypsin/EDTA solution, resuspended as isolated cells, counted with a Thoma slide and used for the in vivo experiments. Postnuclear cell lysates from HACAT and SK Mel-28 were prepared using the following lysis buffer: 0.15 M NaCl, Igepal CA-630 (Sigma-Aldrich) 1 %, 20 mM Tris(hydroxymethyl) aminomethane, complete EDTA-free Protease Inhibitor Cocktail Tablets (Roche, Milan, Italy). Cells were washed twice with PBS (Euroclone, Milan, Italy), then 300 µl of lysis buffer was added and cells were harvested by scraping. Cell lysates were collected and incubated 20 min on ice. Then, the extracts were centrifuged at 14,000*g* for 10 min at 4 °C and the supernatant was collected. The protein concentration was then measured by Bradford's procedure (Bio-Rad protein assay dye reagent concentration, Biorad Laboratories, Hercules, CA) and a cuvette spectrophotometer (Uvikon 860, Kontron Instruments, Zurich, CH). Cell death and viability in growing cells were measured according a published protocol (Aguzzi et al. 2004).

Constructs and plasmids

The expression vectors pSG5-TG-2 plasmid, carrying the cDNA coding for human Transglutaminase type 2, or the empty vector (pSG5 mock) were as previously described (Facchiano et al. 2001). The efficacy of the constructed plasmids was tested by transfecting primary bovine aortic endothelial cells (BAECs) to overexpress TG-2 whose enzymatic activity was measured by labeled polyamine incorporation assay as described (Facchiano et al. 2001). Increased expression of TG-2 was verified by western blot analysis of pSG5-TG-2 transfected cells versus pSG5-mock-transfected cells. For transfection efficiency evaluation, co-transfection with two vectors pSG5-TG-2, and pSG5-GFP, was carried out at the ratio 10:1, using Lipofectamine (Invitrogen) according to published protocols (Sambrook et al. 1989), to allow the internalization of both plasmids by the same cell (Wigler et al. 1979).

SDS-PAGE and western blot analysis

Slab gels (2.4–15 % continuous acrylamide–bisacrylamide gradient vertical gels, manually poured into 16 × 18 cm, thickness 1.5 mm, 15 wells) were generated with a gradient maker (Model 385, Bio-Rad, Hercules, CA, USA) and run with the SE 600 Ruby Apparatus (Hoefer Inc., Holliston, MA, USA), using fresh solutions, as described elsewhere (Facchiano et al. 2010). Western blot analyses were performed using a polyclonal anti-TG-2 (Thermo Scientific, Fremont, CA) at 1:500 dilution and anti-tubulin (Oncogene, La Jolla, CA) at 1:4,000 dilution, followed by chemiluminescent detection as described (Facchiano et al. 2010). Acrylamide, bisacrylamide and 2-beta-mercaptoethanol were from ICN Biomedicals (Irvine, CA, US); $AgNO_3$ was from Merck Eurolab (Lutterworth, Leicestershire, UK). All other chemicals and reagents used, analytical grade, were from Sigma-Aldrich (St. Louis, MO).

Transglutaminase activity assay

For intracellular TG-2 transamidating activity, B16-F10 cells plated on 100-mm Petri dishes (1×10^6) were grown in the presence of [^{14}C]-methylamine (0.5 µl/ml DMEM) as described (Forni et al. 2009). After 48 and 72 h incubation, cells were detached by scraping, washed three times with PBS, and proteins precipitated in 10 % TCA, washed extensively, solubilized in 0.1 N NaOH at 37 °C. Radiolabelled amine incorporation into cell proteins was measured with a scintillation counter (Beckman LS–5000TD, Fullerton, CA).

Animal experiments

Male C57BL/6N mice (6- to 8-week old) were obtained from Charles River Laboratories (Calco, Italy). Lung metastases were produced by intravenous injection of 2×10^5 viable B16-F10 cells, suspended in 0.2 ml DMEM into anesthetised mice (Farmotal, 50 mg/kg body weight).

As a control, a group of animals (normal) was injected with 0.2 ml DMEM only. The viability of the injected cells, controlled by Trypan Blue dye exclusion, was always higher than 95 %. For each experimental condition, 10 mice were inoculated with plasmids in the lateral tail vein at the reported time points. In preliminary experiments, the amount of plasmid injected was 20, 50, 100, 200 µg/100 µl and the dose showing the highest gene transfer efficiency and the lowest toxicity was selected for the following experiments (i.e. 90 µg/100 µl/mice). Mice were killed by cervical dislocation after tumor cell injection for the histological studies. Lungs were rapidly excised, rinsed in phosphate buffered saline (PBS), weighted and further processed. Protocol for theophylline administration of mice was previously described (Lentini et al. 2000a). All experimental protocols have been carried out following the guidelines for the welfare of animals in experimental neoplasia (*Br J Cancer* 1998, 77:1–10) and the ECC Council Directive 86/609, OJL 358, 1 December 1987.

Histology

Samples were fixed in 10 % formalin for 48 h, dehydrated in ethanol and embedded in paraffin. Tissue 10-µm-sized serial sections performed every 100 µm were obtained with a microtome Leitz 1512 (Leitz, Stuttgart, DE) and stained

with haematoxylin and eosin. For the quantitative analysis of the tumors, the number of surface metastases was evaluated by two independent operators. A total of 500 lung foci was considered as the maximum value, and expressed as >500. The precise localization of B16 melanoma colonies in the lungs and the morphometric evaluation were performed on ~100 random lung sections in each lung. Quantification of the portion of lung section occupied by metastatic tissue (percentage of implantation), the growth index (GI) and the invasion index (II) were obtained using an integrated image analysis system (Quantimet 970, Cambridge Instruments Ltd, Cambridge, UK). Densitometric analyses of digitalised microscopical images performed as described (Lentini et al. 2000b) were used to discriminate metastases of B16-F10 melanoma from normal lung tissue. Since the area of a metastatic focus is related to its growth, we considered as GI the ratio between the average area of metastatic foci over the total area of the histological sections. Furthermore, the number of lung foci, related to the invasion of tumor cells, is expressed by II, calculated as the ratio between the total metastatic area and the average area of the metastatic foci.

LC–MS/MS analysis

Peptide mixtures were analyzed by nanoflow reversed-phase liquid chromatography tandem mass spectrometry (RP-LC–MS/MS) using an HPLC Ultimate 3000 (DIONEX, Sunnyvale, CA) connected on line with a linear Ion Trap (LTQ, ThermoElectron, San Jose, CA). Peptides were desalted in a trap column (Acclaim PepMap 100 C18, LC Packings, DIONEX) and then separated in a reverse phase column, a 10-cm long fused silica capillary (Silica Tips FS 360-75-8, New Objective, Woburn, MA), slurry-packed in-house with 5 μm, 200-Å pore size C18 resin (Michrom BioResources, CA). Peptides were eluted using a linear gradient from 96 % A (H_2O with 5 % acetonitrile and 0.1 % formic acid) to 50 %B (acetonitrile with 5 % H_2O and 0.1 % formic acid) in 44 min, at 300 nl/min flow rate. Analyses were performed in positive ion mode and the HV potential was set up around 1.7–1.8 kV. Full MS spectra ranging from m/z 400 to 2,000 Da were acquired in the LTQ mass spectrometer operating in a data-dependent mode in which each full MS scan was followed by five MS/MS scans where the five most abundant molecular ions were dynamically selected and fragmented by collision-induced dissociation (CID) using a normalized collision energy of 35 %. Target ions already fragmented were dynamically excluded for 30 s. Tandem mass spectra were matched against SWISSPROT database and through SEQUEST algorithm incorporated in Bioworks software (version 3.3, Thermo Electron) using fully tryptic cleavage constraints with the possibility to have one miss cleavage permitted, static carbamidomethylation on cysteine residues and methionine oxidation as variable modification. Data were searched with 1.5 and 1 Da tolerance, respectively, for precursor and fragment ions. A peptide was considered legitimately identified when it achieved cross-correlation scores of 1.8 for [M + H]1+, 2.5 for [M + 2H]2+, 3.0 for [M + 3H]3+, and a peptide probability cut-off for randomized identification of $p < 0.001$. Number of identified peptides (#ip) has been considered a semi-quantitative marker of abundance for each identified protein which these peptides belong, as reported in published protocols (Ishihama et al. 2005).

Transglutaminase-substrate bioinformatics analysis

Analysis on known TG-2 substrates to verify their main involvement in human melanoma was carried out by means of the active site collection (ASC) bioinformatic tool specifically developed (Facchiano et al. 2003) available at the following website: http://www.bioinformatics.org/bioinfo-af-cnr/proteomics_tools/. In particular, an updated version of TRANSIT database containing more than 150 known and published TG substrates, with full annotation included, was used.

Statistical analysis

The results were expressed as the mean ± standard deviation (SD) of the values obtained for data point. All experimental data were analyzed by two tails t test (p value lower than 0.05 was considered as significant). For in vivo overexpression experiments, groups of 20 mice were injected with plasmid carrying the TG-2 cDNA or empty vector (mock). Kaplan–Meier survival curves were drawn using the Prism software (Graph Pad Inc., La Jolla, CA).

Results

Transglutaminase-2 overexpression and gene-transfer efficacy

Preliminary experiments were carried out to identify the best protocols to achieve a good gene transfer in a murine model of melanoma metastatic dissemination. The optimal protocols developed are summarized in Fig. 1. As expected, the effect of the plasmid injection was transient, in fact after 7 days TG-2 enzymatic activity dropped back to the basal value (mock). Therefore, to achieve a prolonged transgene overexpression, the plasmid was re-injected in a group of mice on the 7th day after the first plasmid injection, reaching a sustained TG-2 overexpression lasting at

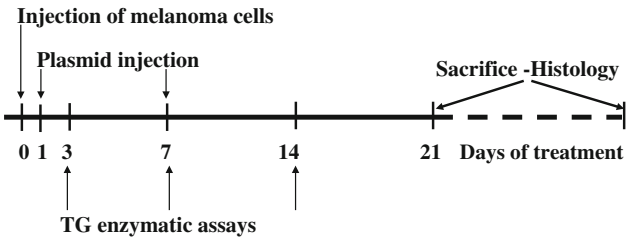

Fig. 1 Experimental protocol to analyze the effects of pSG5-plasmid inoculation on the in vivo TG activity in lungs of B16 tumor-bearing C57BL/6N mice and on metastatic dissemination processes. The plasmid was injected 1 and 7 days after tumor cell inoculation, whereas the enzymatic assays were performed 3, 7 and 14 days after tumor cell inoculation. The histological analyses of dissected lungs were carried out after at least 21 days

least 14 days (Fig. 2a). The plasmids used were pSG5-TG-2 and pSG5-Mock. Total transamidase activity was measured after 3, 7 and 14 days in protein extracts from perfused organs: lung (see Fig. 2a), brain and liver (not shown). In liver and brain, the gene transfer efficiency with this protocol was lower. These data indicated that the developed protocol was able to induce an increase of intratumoral TG-2 enzymatic activity.

Survival studies

The mice survival, upon the described i.v. injections with pSG5-TG-2 and pSG5-Mock plasmids, was also measured at different data points and reported as Kaplan–Meier survival curves (Fig. 2b). The results indicated that the overexpression of TG-2 in melanoma injected mice increased their survival, when compared to mock-transfected mice.

Morphometric studies

In order to understand the mechanisms by which the TG-2 overexpression and increased activity were able to modify the survival rate of melanoma-bearing mice, it was important to discriminate between proliferative or invasive melanoma cell features. At the same time points chosen for the enzymatic assays, the lungs were excised and morphometric analyses were carried out in order to measure the effects on metastasis size and number. As further control, an additional group of mice was treated with 20 mM theophylline solution, according to the protocol previously published (Lentini et al. 2000a), since this compound is able to induce TG-2 activity and melanoma cell differentiation. Morphometric analyses by Quantimet assay were carried out on the histological sections of lungs, to define growth and invasion parameters (see Fig. 3; Table 1). These data indicated that the number of metastatic foci (II) was more markedly modified by the TG-2 overexpression than the metastatic size (GI).

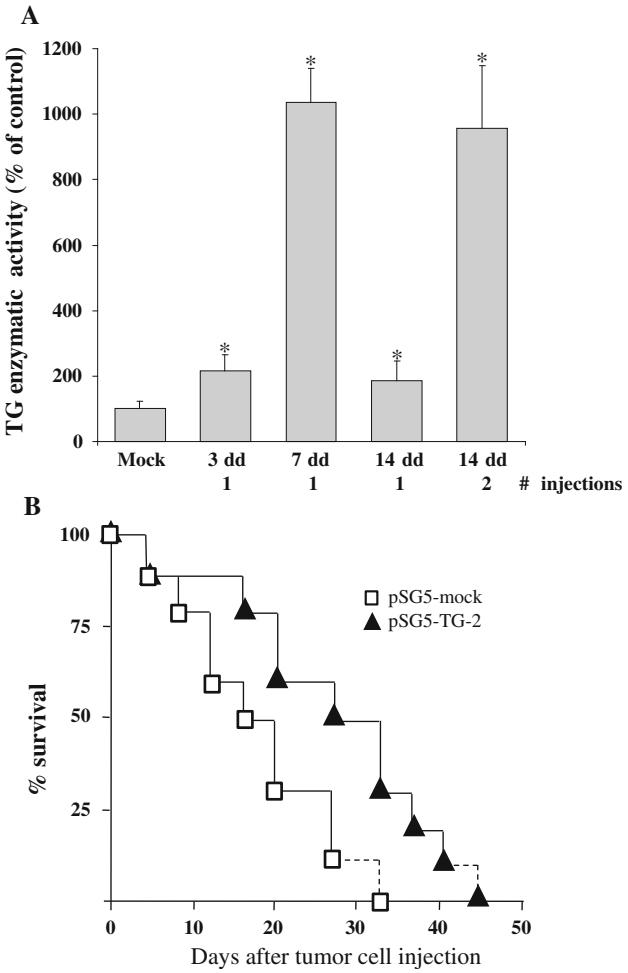

Fig. 2 **a** Gene-transfer efficiency evaluated by quantification of total TG-2 enzymatic activity in tissues from treated mice. Total lung TG-2 activity in mice i.v. injected with pSG5-TG-2 or mock plasmids was measured in protein extracts from perfused organs: lung (shown), brain and liver (not shown). In liver and brain, the gene transfer efficiency with this protocol was lower. The basal TG-2 activity in mock-injected lungs was 29 pmol of incorporated polyamine per mg of protein per hour. Data are plotted as the mean ± SD of three different determinations (statistical significance versus control: $*p < 0.01$). **b** Effect of TG-2 overexpression on the overall survival of melanoma-bearing C57BL/6N mice

Transglutaminase-2 expression in melanoma cell lines

Cell lysates prepared from human SK Mel-28 melanoma cells and human keratinocytes (HACAT), used as control, were analyzed by western blot to measure TG-2 expression (Fig. 4). The enzyme expression was significantly lower in melanoma cells than in control keratinocytes.

Transglutaminase substrates in proteomic analysis of melanoma versus keratinocytes lysates

These results prompted us to analyze the expressed proteomes of both cell types, HACAT and SK Mel-28, which

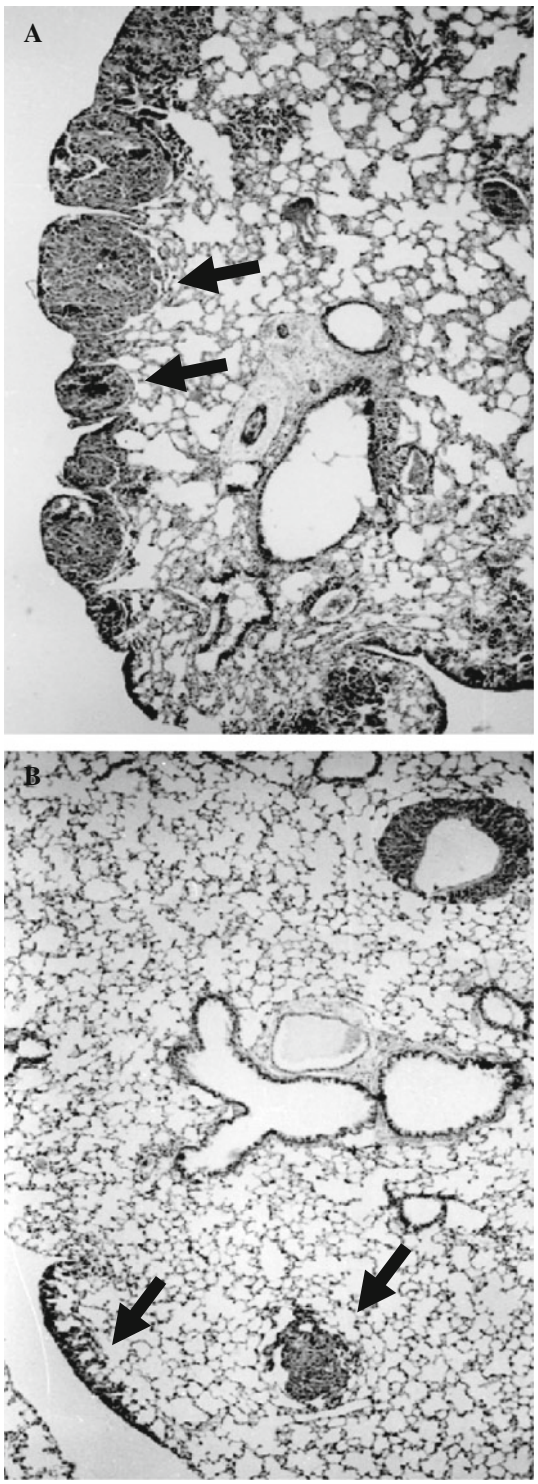

Fig. 3 Histological sections of lung of B16 tumor-bearing C57BL/6N mice injected intravenously with **a** pSG5 plasmid (mock) or **b** pSG5-TG-2 plasmid. *Arrows* indicate melanoma metastases. Original magnification: ×200

were grown in the absence of serum and lysed to analyze their proteomes. Cell lysates were electrophoresed and the whole proteomes analyzed by LC–MS/MS, in order to identify as many as possible expressed proteins, using a high stringency analytical procedure for unambiguous identification. For statistical significance, the experiment was repeated three times. The identified proteomes consisted of 558 ± 122 total proteins in HACAT (HACAT_Proteome) and 552.7 ± 54 total proteins in SK Mel-28 (SK28_Proteome) lysates. The number of total identified proteins was not significantly different ($p = 0.669$). Each one of the identified proteins was then matched against a TG substrate database available online (see "Materials and methods") and the amount of each TG substrate present in both cell lines, calculated as reported in "Materials and methods" ("LC–MS/MS analysis") (Ishihama et al. 2005), was compared. These results are reported in Table 2, showing the relative abundance of 17 TG substrates and the statistical analysis.

Table 1 Morphometric parameters obtained by computer-assisted image analysis performed on histological sections of B16-F10-invaded mice lungs

	Growth index	Invasion index
Saline	0.015 ± 0.001	162.4 ± 13.7
Theophyllin	0.009 ± 0.001	102.4 ± 9.8*
pSG5–mock	0.013 ± 0.002	145.1 ± 11.5
pSG5–TG-2	0.011 ± 0.001	74.5 ± 6.6**

Treatment versus saline: * $p < 0.05$

Treatment versus mock: ** $p < 0.001$

Discussion

The role of TG-2 in tumor progression is still a controversial issue. With the aim to clarify this issue, a direct approach was carried out. In order to achieve an increased TG-2 expression and activity, and to evaluate tumor progression along with mice survival, an in vivo gene-transfer technique was performed. Following i.v. injection in C57BL/6N mice of a plasmid carrying the cDNA for TG-2, the transamidating enzymatic activity was measured in protein extracts from perfused organs: lung, brain and liver (Figs. 1, 2). In lungs, the gene transfer efficiency, measured through the quantification of total TG enzymatic activity, was very high (Fig. 2a), while in liver and brain it was lower (not shown). The mice survival upon the described i.v. injections with pSG5-TG and pSG5-mock plasmids was also measured (Fig. 2b). The results suggested that the overexpression of TG-2, coupled to increased enzymatic activity in lungs of melanoma-bearing C57BL/6N mice, significantly enhanced the survival rate of animals. Enzymatic assays confirmed that the increased expression of TG-2 was coupled to an increased transamidating activity. Morphometric analysis of lungs, performed on plasmid-

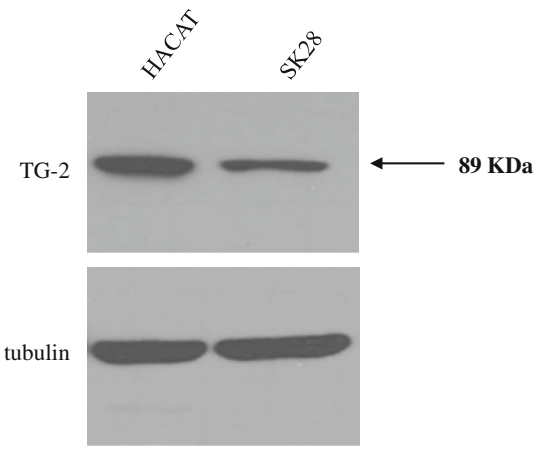

Fig. 4 Western blot analysis of HACAT and SK Mel-28 cell lysates to evaluate the TG-2 expression. Equal amounts of proteins from cell lysates (20 μg) were loaded

treated mice, indicated that the observed higher survival rate was likely related to the reduction of the frequency of metastatic foci, than to a change in their size (Fig. 3; Table 1).

Enzymatic assays reported in previous studies on human keratinocytes (Piacentini et al. 1988) and murine melanoma cells (Forni et al. 2009) showed the presence of a transamidating activity of TG. We are reporting that TG-2 expression appears lower in human SK Mel-28 melanoma cells than that of control keratinocytes (HACAT) (Fig. 4). This evidence was also confirmed in melanoma specimens from patients who underwent surgical excision, where the expression of TG-2 was found very low (data not shown).

The low TG-2 activity in eight human melanoma tumors has also been confirmed by a preliminary analysis using GEO database at http://www.ncbi.nlm.nih.gov, showing that mRNA levels of TG-2 in this neuroectodermal tumor are reduced when compared to lung cancer (9 cases), ovarian (6 cases), prostate (2 cases) and renal (8 cases) cancer. The proteomic study carried out with SK Mel-28 melanoma cell lysates, compared to HACAT control cells, revealed significant differences of expression of known TG-2 substrates, like actin, clathrin heavy chain, galectin 3, several members of the histone family and spectrin alpha. As expected, some members of keratin family were found more expressed, although in not significantly different manner, in keratinocytes than in melanoma cells. It is noteworthy that the total number of proteins identified in the two proteomes was not different (558.0 ± 122 vs. 552.7 ± 54), suggesting that the observed differences between the expression of TG-2 substrates are highly significant. In our knowledge, this represents the first analysis regarding a quantitative evaluation of TG-2 substrates in human melanoma cells compared to a control cell line, using a proteomic approach. Mass spectrometry-based analysis of proteomes of both cell lines indicated that actin, clathrin heavy chain, galectin 3, several histone family members and alpha-spectrin are present in melanoma cells in a significantly higher amount ($p < 0.05$). This suggests a possible role played by TG on cytoskeleton and membrane dynamics, at plasmatic membrane level, as suggested by the higher amount of actin and spectrin. The increased levels of several members of histone family in melanoma cells, compared to keratinocytes, may reflect a higher nuclear instability and/or sensitivity to apoptosis (Singh et al. 2010). More interestingly, we observed an increased expression of galectin 3. This confirms recent findings about the role of galectin 3 in adhesion, angiogenesis, proliferation, differentiation and metastasis in many tumors and whose expression has been recently associated to melanoma progression and prognosis (Brown et al. 2012). It is noteworthy that some of the reported TG-2 substrates were found only in SK Mel-28 cell lysates and undetectable in HACAT cell lysates. The observed highly significant differences in TG substrates expression between melanoma and control cells opens a new point of view to understand the role of TG in cancer: the existence of a balance, within the cell, between TG enzymatic activity and substrate availability. In fact, it seems that normal cells exhibit high expression of TG-2 concomitantly with a low expression and availability of several important TG substrates, compared to melanoma cells. Therefore, changes of this delicate balance (i.e. induction of TG-2 overexpression) may be useful for reduction of cancer progression. The in vivo protective role of TG-2 overexpression is strongly supported by the data obtained in the experimental model of metastasis used, and it has also been confirmed preliminarily by immunohistochemistry in human melanoma specimens, where the enzyme was found localized on the endothelium of intratumoral vessels, or in the microenvironment surrounding the tumor (data not shown).

Even though this study has been focused on just one of the several enzymatic activities of TG-2 (transamidating), it is reasonable to speculate about a possible involvement of other TG activities (e.g. GTP-binding, deaminase, kinase and isomerase) (Facchiano et al. 2006) in cancer development and progression. The overall results of our study indicate an important protective role of TG-2 against melanoma metastatic dissemination, likely via the modulation of the host versus tumor reaction rather than modulation of melanoma cell functions.

As a summary, the reported results indicated that: (a) TG-2 enzymatic activity is protective against in vivo melanoma progression and severity, since its overexpression increases significantly mice survival; (b) TG-2 activity is likely more involved in the processes related to the frequency of metastatic foci (related to adhesion, migration, secretion of MMP secretion, ECM stabilization and

Table 2 Analysis of proteomes identified by mass spectrometry of 2 human cell lines (HACAT and SK Mel-28)

	HACAT (# ip)	SK28 (# ip)	*t* test SK28 vs. HACAT
Actin (cytoplasmic)	15.67	40.67	$P < 0.05$ ←
Alpha-actin	25.00	35.67	$P < 0.24$
Clathrin heavy chain	17.67	75.67	$P < 0.04$ ←
Filamin A	26.00	22.67	$P < 0.79$
Galectin 3	ND	2.00	←
H2A histone type 1	ND	6.50	←
H2B histone type 1	ND	5.50	←
H4 histone	ND	10.33	←
10-kDa heat shock protein	3.00	5.33	$P < 0.33$
60 kDa heat shock protein	28.33	36.67	$P < 0.72$
Keratin, type II cytoskeletal 1	88.67	62.00	$P < 0.54$
Keratin, type II cytoskeletal 2 epidermal	27.67	23.33	$P < 0.75$
Keratin, type II cytoskeletal 5	9.00	2.33	$P < 0.10$
Keratin, type II cytoskeletal 6A	2.00	3.67	$P < 0.51$
Nucleophosmin	0.67	4.33	$P < 0.08$
Spectrin alpha	ND	24.33	←
Vimentin	4.67	43	$P < 0.06$

Proteomic analysis to evaluate the expression of TG substrates in melanoma versus control cells. Starting from the same amount of both cell lysates (90 μg), proteins were separated by gradient SDS-PAGE then the whole proteomes were identified by LC–MS/MS analysis. Such proteomes were then matched to the ASC TRANSIT bioinformatic tool (Facchiano et al. 2003), which contains the sequences and annotations of more than 150 known TGs' substrates. The relative amount of each known TG substrate expressed in HACAT versus SK Mel-28 cells is reported as number of identified peptides (# ip, mean of 3 experiments). Black arrows indicate the known TG substrates whose expression is significantly different between the two cell types. Longer arrows indicate TG substrates present in melanoma cells but not detectable in HACAT cells

angiogenesis) rather than in the size of metastatic foci (related to tumor cell proliferation); (c) the proteome from human melanoma cells is significantly different from the proteome of control human keratinocytes, in particular, regarding the amount of known TG substrates. In this light, the TG-2 involvement in melanoma progression may be seen by a different point of view, opening a new *scenario* involving the balance between the TG enzyme activity and the availability of its substrates.

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