Amino Acids (2009) 36:717–724
DOI 10.1007/s00726-008-0119-5

ORIGINAL ARTICLE

In vivo evaluation of type 2 transglutaminase contribution to the metastasis formation in melanoma

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Received: 6 March 2008 / Accepted: 20 April 2008 / Published online: 2 July 2008

Abstract One of the most relevant problems in tumour treatment resides on the ability of the tumour to form metastasis and disseminate among the organism. The formation of metastases is a complex process, which requires the action of various effectors, not yet completely identified. The analysis of various types of tumours revealed a complex picture about the relationship between type 2 transglutaminase (TG2) expression and outcome and/or metastatic potential of the tumour itself. In some tumours, the transition to a highly invasive state is paralleled by an up-regulation of TG2 expression and/or activity while in some other a down-regulation has been reported. In addition, host tissues seem to react to tumour invasion by up-regulating TG2 expression. In order to analyse whether TG2 might be involved in the metastatic process in melanoma, we studied the metastases formation and development by means of the B16-F10 murine melanoma cell line and with $TG2^{-/-}$ mice as experimental model. Our results indicate that TG2 absence in the host is a favouring condition for the formation and development of the metastasis, while the presence of TG2 in the tumour's cell might be requested for the development of the metastasis.

Keywords Transglutaminase 2 · Melanoma · B16-F10 · Metastasis

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Introduction

Type 2 transglutaminase (TG2) is a multifunctional enzyme, which might act as a protein cross-linking enzyme in programmed cell death; as a G-protein in adreno-receptor signalling; as a protein disulphide isomerase (PDI) in maintaining mitochondrial physiology; and as secreted protein in the stabilisation of the extra-cellular matrix (ECM). The intra cellular cross-linking activity of TG2 needs to be activated by high Ca^{2+} levels paralleled by a decrease of the intracellular GTP levels. In fact, GTP binding shifts the enzyme to a membrane bound rather than to a cytosolic form, revealing its G-protein function as the $G_{(\alpha h)}$ subunit of the α1-adrenergic receptor [for a review see (Fesus and Piacentini 2002; Lorand and Graham 2003)]. Recently, we reported that TG2 might regulate dependent priming of apoptosis at mitochondrial level and possess a protein disulfide isomerase (PDI) activity that is involved in the maintenance of mitochondrial physiology (Rodolfo et al. 2004; Mastroberardino et al. 2006; Malorni et al. 2008). In addition, TG2 might be secreted and localised to the cell surface and in the extra cellular matrix (ECM), where it is able to interact with integrins and integrins binding proteins and contribute to ECM stabilisation (Zemskov et al. 2006).

Melanoma is the deadliest form of skin cancer and its incidence in the world is rapidly growing (Stewart and Kleihues 2003). The malignant transformation of normal melanocytes to melanoma follows four different stages. In stages I and II of the disease, complete surgical excision of the primary tumour is associated with a success rate of about 95%. However, in stage III (lymph node infiltration) and stage IV (distant metastasis), when melanoma has disseminated to multiple organs, including brain, lungs, liver and bone, surgical interventions are of limited use.

Moreover, systemic therapy has minimal success because of the high intrinsic resistance exhibited by melanoma cells against various anticancer therapies. A large number of genetic, functional, and biochemical studies suggest that melanoma cells become resistant to chemotherapy by exploiting their intrinsic resistance to apoptosis (Miller and Mihm 2006; Crowson et al. 2007). Alterations that contribute to the development of resistance to apoptosis can enable cancer cells not only to develop resistance to drugs but also to survive under stressful conditions (e.g. during metastasis) (Fesik 2005). The elucidation of novel proteins and pathways involved in the development of apoptosis' resistance as well as the comprehension of their contribution to the metastatic process may reveal promising molecular targets for effective treatment of cancer.

During the last two decades, various reports have dealt with the relationship between TG2 expression and cancer development. The picture arising from these observations is that tumour cells not only have altered expression levels of TG2, with respect to their normal counterparts, but also might contain modified TG2 forms, which are sometimes inactive, and/or may differ in sub-cellular localisation. Tumours usually display high TG2 activity in the cell particulate fraction although the absolute amount of enzyme present in this fraction is normally not altered (Barnes et al. 1985; Knight et al. 1990a, b; Beninati et al. 1993; Tunici et al. 1999). The decline of TG2 activity in tumours is potentially a bad prognostic marker and is possibly related to the metastatic potential of the tumour (Jiang et al. 2003a, b; Mehta et al. 2004). It has been observed that changes in the TG2 contents and activity might be related to the onset of chemo-resistance as well as to the ability of the tumour to metastasize. Given the proposed functions of TG2, a reduced expression and/or enzyme activity in tumours would indeed lead to a resistance to apoptosis induction as well as to a less stable surrounding ECM, resulting in a reduction of cell adhesion and an increase of the migration rate, thus facilitating the initial invasive stage of the tumour. However, there are reports of increased TG2 expression in highly invasive tumours, for example in the breast, and an increase of TG2 expression has been reported in various secondary metastatic tumours. Conversely, host tissues frequently display high levels of TG2 expression and activity, especially at the host-tumour interface. This particular event has been interpreted as a sort of host defence system against the tumour and might act to inhibit tumour growth.

In addition, TG2 protein expression and activity result to be very high in the endothelium of blood vessels, and it could be hypothesized that TG2 plays a role not only in the stabilisation of the ECM, but also in the rearrangement and neo-formation of vessels in the metastatic process.

In order to address, at least, some of these points we take advantage of the $TG2^{-/-}$ mice model (De Laurenzi and Melino 2001) to study the process of primary melanoma tumours development and metastasis formation in vivo.

Materials and methods

Cell culture

Highly metastatic murine melanoma cells, B16-F10 were propagated in D-MEM medium supplemented with 10% foetal bovine serum (Invitrogen), at 37°C under 5% CO_2. The spontaneous and experimental metastatic activity of this line was evaluated and found stable during the time of the experimental procedure. Cell viability was tested by the Trypan Bleu (0.25%) exclusion test at different interval times (24, 48 and 72 h).

Western Blotting

Whole cell lysate (15 μg), separated on 4–12% Nu-PAGE pre-cast gel (Invitrogen) and transferred onto a nitrocellulose membrane, was probed with anti-TG2 rabbit polyclonal (1:500, Ab-4; Neomarkers) or anti-TG2 polyclonal (1:500, T7066, Sigma). Secondary anti-rabbit and anti-goat horseradish peroxidase conjugated antibodies were from Jackson Immunoresearch. Chemiluminescence was revealed by means of FluorChem System (Alpha Innotech).

Primary tumours and experimental lung metastases

The B16-F10 murine melanoma cell line is the commonly used model to test in vivo formation of tumours and metastases in C57Bl/6 mice. This syngeneic cell line easily developed primary tumours when injected subcoutaneously in the animals flank and, due to their high invasiveness, are able to induce metastasis formation in the lungs after injection into the caudal vein. To assess the formation of primary tumours, C57Bl/6 mice were inoculated into the right flank with 5×10^4 B16-F10 cells and suspended in 0.1 ml sterile PBS. The growth of the tumour was monitored by means of calliper measurement, and tumour volumes were calculated using the formula 0.4 ($A^2 \times B$), where B represents the largest diameter and A the diameter perpendicular to B. At day 20, the animals were sacrificed, the tumour explanted, measured and fixed for subsequent histological examination.

For the experimental lung metastases formation, B16-F10 cells, cultivated in vitro are used for the assay when in log phase. We treated 120 mice for each genotype, 40 mice for each time point, by injecting 2×10^5 viable cells,

suspended in 0.1 ml sterile PBS, into the caudal vein of the animal and the analysis of metastasis formation rate was conducted at 10, 15 and 20 days post-injection (p.i.). The mice were sacrificed by cervical dislocation and the lungs removed, washed in PBS, fixed in formalin and then metastasis number was assessed by counting under a dissection microscope.

Animals were farmed, housed and experimental protocols carried out following the guidelines proposed by the Italian National Research Council (CNR) and the MiUR as well as by the Istituto Superiore di Sanità (ISS).

Histology

Sample were fixed in 10% formalin for 48 h, dehydrated in ethanol and embedded in paraffin. Serial tissue sections (5 μm) were incubated for 1 h at room temperature with anti-TG2 polyclonal antibody Ab-4 diluted 1:100, followed by incubation with secondary biotinilated-multilink (BioGenex) and then reaction developed with AEC reagent under a microscope.

Invasion assay

Invasion assays were carried out in Boyden Chamber as previously described (Albini et al. 1987). Polycarbonate polyvinylpyrrolidone-free membranes (8 μm pore size, Neuroprobe, Cabin John, MD, USA) were coated with Matrigel, Laminin or Fibronectin (0.5 mg/ml). Cells were harvested, counted and suspended in D-MEM/0,1%BSA at the final concentration of 10^6 cells/ml. Then, 2×10^5 cells were plated onto each filter. 3T3 murine fibroblast conditioned medium was used as chemo-attractant. Cells were allowed to migrate for 5 h at 37°C in a humidified atmosphere containing 5% CO_2. The membrane was fixed in ethanol for 30 s and stained with toluidine blue 2% for 10 min Non-migrating cells on the upper side of the membrane were mechanically removed. The number of invasive cells was evaluated by means of Image J software.

Results

Analysis of primary tumours and metastases formation in $TG2^{-/-}$ versus $TG2^{+/+}$ mice

In order to test whether there was any difference in the formation's rate of primary tumours and metastases between $TG2^{-/-}$ and $TG2^{+/+}$ mice, we use the syngeneic cell line B16-F10. This highly metastatic murine melanoma cell line develops primary tumours, when injected into the flank of the animal, while it induces metastases formation in the lung, when injected into the caudal vein. We investigated the effect of TG2 absence on the formation of primary tumours by means of subcutaneous injection of 5×10^4 B16-F10 cells into the right flank of the animals. Tumour development was followed by calliper measurement every other day, starting from the day of appearance until day 20. We observed an earlier appearance and a faster growth of the tumours in $TG2^{-/-}$ mice, as compared to the wild type animals. Figure 1a shows the representative smaller and bigger tumours developed in $TG2^{+/+}$ (left panel) versus $TG2^{-/-}$ mice (right panel). It appears clearly visible that the tumours developed in $TG2^{-/-}$ mice were bigger than the ones developed in $TG2^{+/+}$ mice, with a volume ratio of about threefold. It is worth to note that the bigger tumour developed in $TG2^{+/+}$ mice is always smaller than the smaller ones developed in $TG2^{-/-}$ mice. In addition, tumours developed in the $TG2^{-/-}$ mice showed a reduction of the connective capsule surrounding the tumour. These results suggested that TG2 might play a role in the confinement and development of primary tumours, by acting on the extra-cellular matrix and not on the tumour itself.

In order to analyse if the absence of TG2 had any influence on lung metastases formation, we treated 120 mice for each genotype (40 mice for each time point), by injecting 2×10^5 cells into the caudal vein of the animals. The analysis of metastases formation rate was conducted at 10, 15 and 20 days post-injection (p.i.). The mice were sacrificed and the lungs, as well as other organs showing metastases formation, were removed, washed in PBS, fixed in formalin and then metastases number was assessed by counting under a dissection microscope. Figure 1b shows a representative set of images of lungs from wild type and TG2 knockout mice, at the indicated time points, and Fig. 1c shows the quantification of the metastases counted by three independent scientists. Interestingly, $TG2^{-/-}$ mice show an early appearance of the metastases and, at each time point, developed more metastasis with respect to $TG2^{+/+}$ ones. This effect, already visible at day 10 p.i., seems to peak at day 15, while at day 20 the ratio between metastases in $TG2^{-/-}$ versus $TG2^{+/+}$ mice decreases, as if the $TG2^{-/-}$ mice were able to eliminate the tumours. We could hypothesise two different but not mutually exclusive explanations for this observation. In first instance, the observed decrease in the number of metastases could be due to an effective loss of the metastases themselves, linked to a defect in the neo-vascolarisation of the growing tumour in the $TG2^{-/-}$ mice. This hypothesis is supported by the high levels of TG2 expression observed in the endothelium of the vessels and by its contribution to their stability, formation and rearrangement (Bikfalvi 1995; Haroon et al. 1999a, b; Bakker et al. 2006; Jones et al. 2006). Moreover, during injection into the caudal vein, we observed an extreme fragility of the vessel in $TG2^{-/-}$ mice.

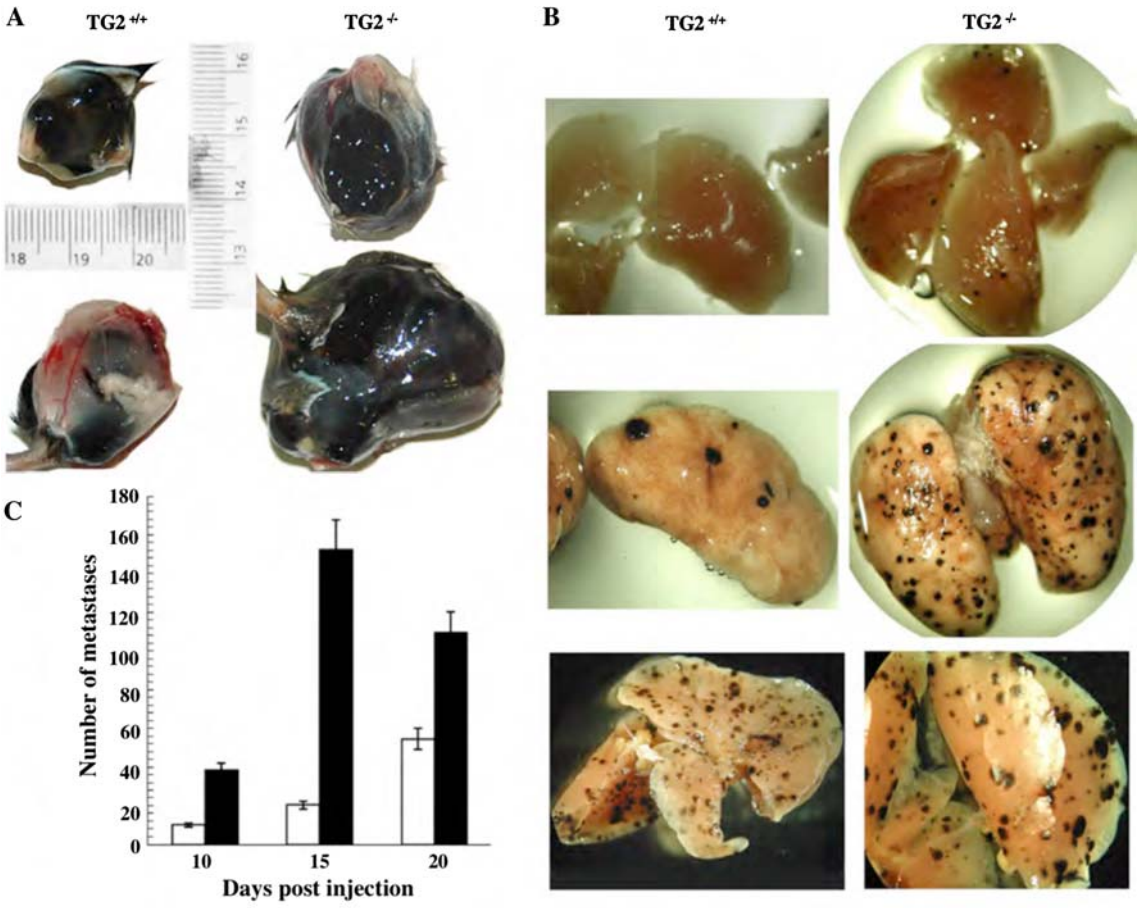

Fig. 1 Analysis of primary tumours and metastases formation in $TG2^{+/+}$ vs. $TG2^{-/-}$ mice. **a** Representative sizes of primary tumours developed in $TG2^{+/+}$ (*left panel*) and $TG2^{-/-}$ (*right panel*) mice at 20 days post subcutaneous injection in the right flank. *Upper panels* show the smallest developed tumours, while the *lowest panels* show the bigger ones. **b** Representative morphology and dissemination of lung's metastases at the indicated times post-injection in the caudal vein. **c** Quantification of the lung's metastases counted by three independent operators, 40 animals for each genotype, for each time point

This observation suggests that the absence of TG2 could be crucial for both the stability and the formation of new vessels in the developing tumours, which then are not able to correctly feed and so they die. The other hypothesis is based on the morphological observation of metastases evolution and progression at 10, 15 and 20 days p.i. in $TG2^{-/-}$ vs. $TG2^{+/+}$ mice (Fig. 1 panel b). At day 10, both $TG2^{-/-}$ and $TG2^{+/+}$ mice developed small size metastases (micro-metastases), even if the number in $TG2^{-/-}$ mice is about fourfold higher (Fig. 1c). At day 15, $TG2^{+/+}$ mice showed about sevenfold less metastases but they appear to be bigger when compared with that of the $TG2^{-/-}$, which still have a lot of micro-metastases and some bigger ones. At day 20, the size of the metastases seems to be comparable in $TG2^{-/-}$ and $TG2^{+/+}$ mice, even if $TG2^{-/-}$ mice showed about twofold more metastases with respect to $TG2^{+/+}$. If the "normal" metastases' development follows the pathway observed in wild type mice, what we observed in the $TG2^{-/-}$ mice at days 10 and 15 might be a proneness to form metastases. This early metastases appearance, as well as the speeding up of the process, could rely on an increased extravasation rate of the injected B16-F10 cells. The lack of TG2 might alter the stability of the extracellular matrix (ECM) and facilitate both the extravasation process from the vessels as well as the migration through a less dense ECM. Nevertheless, at day 20 $TG2^{-/-}$ mice still show about twofold more metastases with respect to the $TG2^{+/+}$ mice, indicating that TG2 absence might be important in the overall process of metastases formation in melanoma. In addition, $TG2^{-/-}$ mice frequently show the presence of metastases in organs other than lungs, such as kidney, liver and spleen, again suggesting that the lack of TG2 might be a favourable condition in those organs where there is a strong blood flow.

Effect of TG2 expression in B16-F10 cell line

The data obtained so far suggested that host TG2 might play a role in the metastases formation, either at vessel or ECM level, but does not give any indication about a

possible implication of the TG2 expressed by the tumour's cells. In order to get further insight on this aspect, we analysed the TG2 expression levels in the murine melanoma B16-F10 cell line. The analysis of TG2 expression in human derived cell lines does not represent a problem, thanks to the good quality of the commercially available antibodies. On the other hand, TG2 expression in murine cells results to be quite difficult because all commercially available antibody against TG2, showed poor specificity and might lead to misinterpretation of the results (Rodolfo et al. 2008). Figure 2a shows a representative western blot of B16-F10 cell extract, compared to positive ($TG2^{+/+}$) and negative ($TG2^{-/-}$) controls. Even if both the antibody used (Ab-4, Neomarkers; T7066, Sigma) cross-react with a non-specific protein, very close to TG2, the Ab-4 antibody seems to be the most effective in recognising the murine TG2. This result clearly indicates that cultured B16-F10 cell line does not express TG2 at detectable level.

We used then this antibody in a series of immuno-histochemistry experiments, in order to assess if there was any variation in the expression of TG2 in the lung metastases. As visible in Fig. 2b, in the lung's sections belonging to $TG2^{+/+}$ mice (left side), the antibody stains both the parenchyma as well as the metastases, while in the lungs of $TG2^{-/-}$ mice, the staining seems to be restricted only to the metastases and to the ECM surrounding the metastases themselves (right side). We must be very careful when drawing results from these observations because we must take in account that all the commercial antibody do recognise a non-specific protein in western blot, so they might recognise also something else in immuno-histochemistry. Our data suggest that B16-F10 cells does not express TG2 in culture but begin to express the protein once they start to form metastases. In addition, we observed that TG2 expression is proportional to the increase of the dimensions of the metastases. The up-regulation, as well as the down-regulation, of TG2 gene expression has been reported for various tumour derived cell lines, in relationship with their metastatic potential and this is an aspect not yet completely clarified (Barnes et al. 1985; Hand et al. 1987; Knight et al. 1990a, b, 1991; Zirvi et al. 1991; Mehta 1994; van Groningen et al. 1995; Haroon et al. 1999a, b; Tunici et al. 1999; Jiang et al. 2003a, b; Verma et al. 2006; Xu et al. 2006; Lentini et al. 2007; Satpathy et al. 2007; Verma and Mehta 2007; Xu and Hynes 2007).

It has been widely reported that TG2 induction might lead to cell death but very recently there have been reports about the pro-survival role exerted by TG2 in breast cancer (Oliverio et al. 1999; Piacentini et al. 2002; Antonyak et al. 2006; Herman et al. 2006; Mangala et al. 2007). We analysed whether TG2 induction sensitised B16-F10 cells to staurosporine-induced cell death. In order to induce TG2 expression, B16-F10 cells were treated with all-trans retinoic acid (RA) 20 μM for 96 h and then TG2 expression was assessed by Western blot on whole cell extracts. Figure 2c, shows a representative blot of RA-dependent TG2 induction and Fig. 2d the effect of TG2 expression on

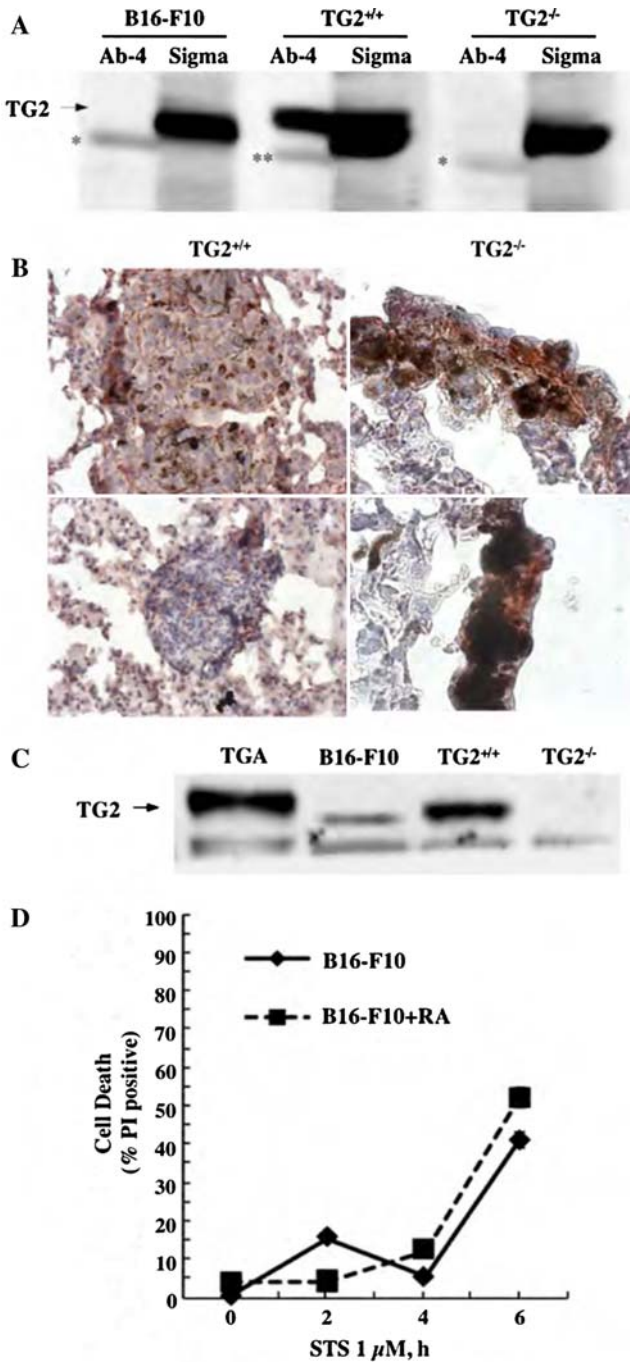

Fig. 2 **a** Analysis of TG2 expression in B16-F10 cell line. a B16-F10 whole cell extracts compared to $TG2^{-/-}$ and $TG2^{+/+}$ livers protein extracts. * and ** indicate non-specific cross-reacting proteins. **b** Immuno-histochemistry on lung tissue slides with Ab-4 antibody. **c** RA-dependent TG2 induction in B16-F10 cell line. **d** Analysis of staurosporine induced cell death in B16-F10 cells expressing TG2

STS induced cell death. These results indicate that RA-dependent induction of TG2 expression slightly sensitised B16-F10 cells towards cell death through the mitochondrial pathway of apoptosis. In the light of these results, the increased expression of TG2 in the metastasis might lead to two different and opposite effects. On one side, TG2 expression and secretion might favour ECM rearrangements, on the other side might be deleterious for the survival of the metastasis itself, as indicated from the slight amount of cell death rate observed.

TG2 dependent cross-linking of ECM reduces the invasiveness of B16-F10 cells

It has been recently demonstrated that TG2 dependent modification of ECM inhibits tumour invasion (Mangala et al. 2005; Jones et al. 2006) and TG2 expression modifies drug resistance of breast cancer (Mehta 1994; Herman et al. 2006; Mangala et al. 2007). Our observations suggest that TG2 might be implicated in the melanoma's metastasis formation in two different ways. On one side, the lack of TG2 in the host might favour the induction of the metastatic process, while the induction of TG2 expression in B16-F10 metastases might be requested by the metastasis itself to develop and grow.

In order to clarify the role played by TG2 and its cross-linking activity in the stabilisation of ECM and the connective tissue surrounding the tumour, we performed an in vitro migration assay (Albini et al. 1987) with different substrates pre-treated with enzymatic active or inactive TG2. Figure 3 shows how B16-F10 migration is influenced by TG2 cross-linking activity on different matrigel (M), laminin (L) and fibronectin (F) substrates, which mime ECM. It is interesting to note that, even if at different extents, TG2 enzymatic activity on ECM components always reduces the number of cells able to migrate through the cross-linked substrate. These observations support the hypothesis that a lack of TG2 might result in a less dense ECM, a favourable condition for both extravasation of circulating cells as well as for the migration and development of metastases.

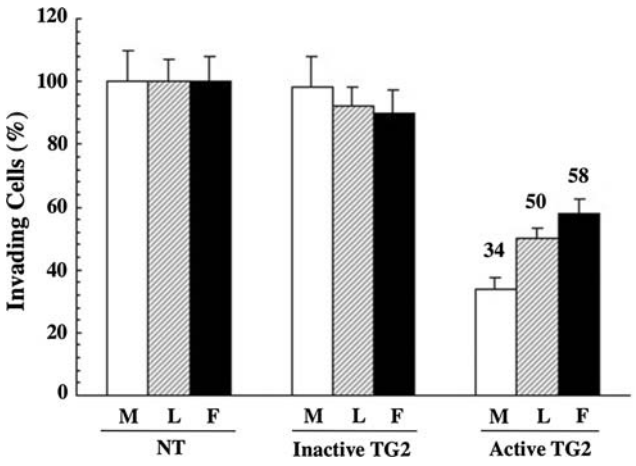

Fig. 3 Migration rates of B16-F10 cells on different ECM-mimicking substrates. Matrigel (*M*), Fibronectin (*F*) and Laminin (*L*) coating substrates were pre-treated with either enzymatic active or inactive TG2, before performing the invasion assay

Discussion

Type 2 Transglutaminase (TG2) is an ubiquitary enzyme whose expression has been frequently linked to cell death induction (Oliverio et al. 1999; Fesus and Piacentini 2002; Piacentini et al. 2002; Rodolfo et al. 2004). During the last two decades, the analyses of various tumours-derived cell lines delineated a very complex pictures about TG2 expression and the ability of tumours to develop and form metastases (Barnes et al. 1985; Hand et al. 1987; Knight et al. 1990a, b, 1991; Menter et al. 1991; Zirvi et al. 1991, 1993; Beninati et al. 1993; Mehta 1994; Hager et al. 1997; Hilton et al. 1997; Haroon et al. 1999a, b; Jiang et al. 2003a, b; Mangala et al. 2005, 2007; Fok et al. 2006; Herman et al. 2006; Jones et al. 2006; Verma et al. 2006; Xu et al. 2006; Verma and Mehta 2007; Xu and Hynes 2007; Yuan et al. 2007). In fact, not all the tumours behave and display the same features, with differences in TG2 expression that could account for an increase in the resistance of the primary tumour and for the metastatic potential as well as for a reduction of both these aspects. It has been reported that high levels of TG2 expression in non-metastatic primary tumours decrease during the transition to highly metastatic forms, suggesting that low TG2 protein or activity levels might lead to the alteration of the adhesion properties of the cancer cell and of the stability of the ECM in the neighbourhoods of the tumour. These conditions might favour the detachment of cells from the primary tumour and their migration in the blood stream. On the other hand, there are reports about induction of TG2 expression in primary tumours, before the transition to a highly metastatic state. In this case, TG2 might function as a pro-survival factor by localising at nuclei level (Antonyak et al. 2001, 2004; Boehm et al. 2002) or at cell surface, where it is able to interact with integrins and stimulate survival signals (Fok et al. 2006; Herman et al. 2006; Mangala et al. 2007). In addition to all these confusing observation, researcher must take in account that tumours belongs to different embryonic lineages, so this might also account for the various observed discrepancies.

Most of these observations take into consideration only the role played by the TG2 expressed by the tumour itself, and only few of them deal with the role that might be

exerted by TG2 expressed from the host (Haroon et al. 1999a, b).

Our in vivo observations do not clarify the picture completely, but might be helpful in seeding light on some aspects of the metastatic process in the melanoma animal model. It is important to take into consideration that we are working in an animal model that could differ a lot from humans. In fact, our analysis of B16-F10 cell line showed that, even if highly metastatic, those cells do not express TG2 at detectable levels, in contrast to most of the highly metastatic human melanomas which express TG2 at high levels. It is interesting to note that B16-F10 cells start to express TG2 once they form lung's metastases and that this expression seems to be related to the dimension of the metastases themselves. As for the onset of lung's metastases, $TG2^{-/-}$ mice showed an early appearance of micro-metastases, which developed rapidly and in a quite different way from the wild type mice. The monitoring of metastases development during 20 days showed that the number of metastases developed in knockout mice is always higher than that developed in wild type ones, even if with some variations. In addition, the evolution of the observed micro-metastases seems to be different between the two strains and might be related to the lack of TG2. In fact, $TG2^{-/-}$ mice might present to the growing metastasis a favourable environment since the beginning (e.g. less dense ECM) but then requires the action of tumour's expressed TG2 in order to activate pro-survival signals and/or neo-angiogenesis or vessel rearrangements. As for the $TG2^{+/+}$ mice, the first steps of this process might be more difficult, because of the presence in the host of a TG2-stabilised ECM, while the vascolarisation events might follow the same pathway in both the cases. A further indication that suggests a role for the TG2-stabilised ECM might come from the observation of the primary tumours. The tumours developed in $TG2^{-/-}$ mice are about three-fold bigger with respect to the ones developed in $TG2^{+/+}$ and the connective capsule surrounding these tumours is less visible. An additional confirmation about this role of TG2 in the stabilisation of ECM and on the inhibition of cell migration in our melanoma in vivo model arises from the in vitro migration assays performed with different TG2-modified substrates. We observed that all the three substrate used, matrigel, fibronectin and laminin, does not allow passage of B16-F10 cells when pre-treated with cross-linking active TG2, supporting the already proposed hypothesis of a protective role for TG2 expressed by the host (Haroon et al. 1999a, b).

We examined also, whether the observed induction of TG2 in lung's metastases might exert a pro-death or a pro-survival effect on this cell line. The retinoic acid-dependent induction of TG2 expression does not alter the sensitivity of these cells to cell death induction through the mitochondrial pathway of apoptosis, suggesting that TG2 functions other than the ones exerted in cell death might be involved in this case.

Our data suggest the existence of different contributions by the various TG2 activities to the metastatic process in mice melanoma, and open the question, which of these activities might be modulated or inhibited in order to achieve positive results in melanoma treatment.

Acknowledgments This work was supported by grants from: Associazione Italiana per la Ricerca sul Cancro, to M.P.; MiUR-PRIN 2006, to C.R. and M.P.; Telethon, Grant N. GGP06254 to M.P.

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