EXPRESSION OF TISSUE-TYPE TRANSGLUTAMINASE CORRELATES POSITIVELY WITH METASTATIC PROPERTIES OF HUMAN MELANOMA CELL LINES

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In this study the relationship between tissue-type transglutaminase (TGase2) activity and the propensity to metastasize was investigated in human melanoma cell lines with different metastatic behavior. TGase2 catalyzes an acyl-transfer reaction between peptide-bound glutamine residues and primary amines, including the ε-amino group of lysine residues. Northern-blot analysis demonstrated that TGase2 RNA-expression (3.7 kb) was elevated in highly metastatic cell lines (MV3 and BLM) as compared to weakly metastatic ones (1F6 and 530). Immunoprecipitation and enzyme assays of TGase2 showed that the differential expression at the mRNA level was also reflected at the protein level. These findings reveal a positive relation between the expression of TGase2 and the metastatic properties of the human melanoma cell lines.
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Modifications of proteins commonly serve special purposes, *e.g.* they can modulate the activity of signal transduction cascades, determine protein fate, and stabilize intra- and extracellular matrices. Calcium-dependent acyl transferases, also known as transglutaminases (TGases), form one group of enzymes involved in these processes. TGases (EC 2.3.2.13) catalyse an acyl transfer reaction between peptide-bound glutamine residues and primary amine groups. As a result, substrate proteins incorporate amines (such as polyamines), or are cross-linked via ε(γ-glutamyl)lysine bridges if the amine is a peptide-bound lysine (Folk and Finlayson, 1977).

In mammals, 5 distinct TGases have been described. TGase1 is a particulate or membrane-associated activity of about 90 kDa, originally discovered in epidermal keratinocytes (Thacher and Rice, 1985) and now known to be widely expressed (Kim *et al.*, 1992); TGase2 is a ubiquitous soluble or tissue-type activity of about 80 kDa, which is widely distributed in human cells and tissues (Gentile *et al.*, 1991); TGase3, known as the epidermal or hair follicle TGase, is a soluble pro-enzyme activity of about 77 kDa (Kim *et al.*, 1991); furthermore, an inactive TGase-like protein of about 75 kDa exists, which is a ubiquitous constituent of the plasma membrane of most eukaryotic cells (Sung *et al.*, 1990), and the 77-kDa catalytic subunit of blood-clotting factor XIII also has TGase activity (Ichinose *et al.*, 1986). TGase2, by far the most abundant of these enzymes, has been implicated in the activation of receptor-mediated endocytosis (Davies *et al.*, 1980), hormone secretion (Bungay *et al.*, 1986), cell adhesion by cross-linking of extracellular proteins (Gentile *et al.*, 1992; Martinez *et al.*, 1989), and the formation of the apoptic envelope in cells undergoing programmed cell death (Fesus *et al.*, 1987; Alaoui *et al.*, 1992).

The present study was undertaken to examine the TGase2 activity in human melanoma cell lines with differing metastatic potential. Whereas reports on mouse melanoma cell lines (Beninati *et al.*, 1993), hamster fibrosarcoma (Knight *et al.*, 1990), and rat hepatocellular carcinoma (Hand *et al.*, 1988) suggested that decreased levels of TGase2 activity are associated with increased metastatic potential, our investigations on human melanoma cell lines show that TGase2 expression correlates positively with the gain of metastatic properties.

MATERIAL AND METHODS

Material

All radiolabeled chemicals and Hybond N-plus membranes were from Amersham (Den Bosch, The Netherlands). Kodak X-Omat AR5 films (Rochester, NY) were used for autoradiography. Purified guinea-pig tissue TGase was supplied by Sigma (Brussels, Belgium). Cell culture media and FCS were obtained from GIBCO (Grand Island, NY). All other reagents were of analytical grade.

Melanoma cell lines and xenografts

Human melanoma cell lines MV3, BLM, 530 and 1F6 (van Muijen *et al.*, 1991*a, b*) were grown as monolayers on Dulbecco's modified Eagle's medium supplemented with 10% FCS, glutamine (2 mM), penicillin G (100 units/ml), streptomycin (100 μg/ml) and pyruvate (1 mM). Cells were harvested by trypsinization. Xenograft tumors from nude mice were obtained as described (van Muijen *et al.*, 1991*a, b*). Within the panel of human melanoma cell lines, 1F6 and 530 represent the non-metastatic or weakly metastatic cell lines, with a metastasis frequency of less than 10%. The highly metastatic cell lines BLM and MV3 disseminate with a frequency of 50 and 90%, respectively.

RNA isolation and Northern-blot analysis

Total RNA was isolated using the lithium-urea procedure described by Auffray and Rougeon (1980). Between 10 and 20 μg of total RNA were glyoxylated (McMaster and Carmichael, 1977), size-fractionated on 1% agarose gels and blotted to Hybond N+ membranes (Amersham, Aylesbury, UK). Hybridizations were performed according to Church and Gilbert (1984); the RNA was hybridized overnight in 0.5 M Na$_2$HPO$_4$, pH 7.2, 1% BSA, 7% SDS, 1 mM EDTA and 0.1 mg/ml denatured herring sperm DNA at 65°C with ^{32}P-labeled DNA probes to a maximum of 1 × 10^6 cpm/ml. Filters were washed at 65°C in 0.1 M Na$_2$HPO$_4$, pH 7.2, 0.5% SDS, 1 mM EDTA.

DNA probes

The human TGase2 cDNA, a 3.5-kb EcoR1 fragment, was a generous gift from Dr. P. Davies, Houston, TX (Gentile *et al.*, 1991). A 28S ribosomal probe was used to reconfirm equal loading of RNA samples in each lane. Probes were radiolabeled by nick-translation.

Cytoplasmic extract preparation

Cells were resuspended in 5 ml lysis buffer (10 mM TrisHCl, pH 8.0, 1.5 mM MgCl$_2$, 0.5 mM dithiothreitol, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% trasylol) and lysed by 7 strokes in a Dounce homogenizer. Nuclei were removed by centrifugation at 1,000 g for 15 min. Supernatant was concentrated by 75% (NH$_4$)$_2$SO$_4$-precipitation followed by centrifugation at 150,000 g for 20 min. The pellet containing

the TGase2 protein was resuspended in, and dialyzed against, 50 mM Tris-HCl, pH 8.0, 100 mM NaCl. The protein concentration was determined by the Coomassie brilliant blue binding method (Bradford, 1976).

Enzyme assays

The enzymatic incorporation of [^{14}C]methylamine into endogenous substrate was used to assay TGase activity (Lorand *et al.*, 1981). The incubation mixture contained 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM CaCl$_2$, 10 mM dithiothreitol, 0.4 mM [^{14}C]methylamine with specific activity 56 mCi/mmol and 0.3 mg cytoplasmic protein extract in a final volume of 50 μl. After 60 min incubation at 37°C, 10-μl aliquots of the incubation mixture were spotted onto Whatman 3MM filter paper. Free [^{14}C]methylamine was eliminated by washing with excess of ice-cold 5% TCA. Filters were dried and counted after addition of 5 ml Aqualuma Plus (LUMAC LSC, Groningen, The Netherlands) in a Beckman model 2800 liquid scintillation counter.

For identification of the TGase substrate(s), 5 μl (30 μg) of the incubation mixture were analyzed on 12.5% SDS-polyacrylamide gel. The gel was fluorographed, dried and exposed.

Cell labelling and immunoprecipitation

Cell labelling and isolation of proteins were based on the method described by Banks-Schlegel and Harris (1983). For labelling, the cells were plated in dishes 10 cm in diameter and grown until 50% confluent. Cells were washed twice with PBS and incubated for 30 min with 4 ml of methionine-free Eagle's minimal essential medium, to which were added 40 μCi [^{35}S]methionine (> 1000 Ci/mmol) and dialyzed FCS at a final concentration of 6%. After overnight incubation at 37°C, the cells were washed 3 times with PBS. The labelled cell pellet was lysed in 75 μl of lysis buffer (20 mM Tris-HCl, pH 7.7, 1 mM phenylmethylsulfonyl fluoride, 2% SDS, 10 mM dithiothreitol). The lysates were boiled and centrifuged to eliminate insoluble materials. The supernatant was diluted in 1.425 ml of RIPA buffer without SDS (RIPA buffer: 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.5% Trasylol), and centrifuged. The resulting supernatant was used for immunoprecipitation. Aliquots containing 2.5 × 10^6 cpm of [^{35}S]methionine-labeled cell extracts were used for each precipitation. Affinity-purified, rabbit anti-human TGase2 IgG-type antibodies (Dr. L. Fesus, Debrecen Medical School Apoptosis Research Foundation, Hungary) were used in the immunoprecipitations. After 3 washes with RIPA buffer, the immunoprecipitates were analyzed on a 10% SDS-polyacrylamide gel. The gel was fluorographed, dried and exposed.

RESULTS

Presence of TGase2 transcripts in human melanoma cell lines

Total RNA isolated from different human melanoma cell lines was subjected to Northern-blot analysis with a 3.5-kb human Tgase2 cDNA probe. Autoradiography revealed the cognate, 3.7-kb hybridization signal (Fig. 1). Pronounced Tgase2 expression was seen in the highly metastatic cell lines BLM and MV3 (lanes C, D), while for the weakly metastatic cell lines, only trace expression was detectable in 1F6 (lane B), and no expression in 530 (lane A). To determine whether a corresponding expression pattern would occur in the xenograft tumors of these cell lines, we analyzed total RNA from xenografts (Fig. 2). The observed Tgase2 expression was highest in the BLM- and MV3-derived xenografts (lanes C, D), while no expression was detected in the 530- and 1F6-derived xenografts (lanes A, B).
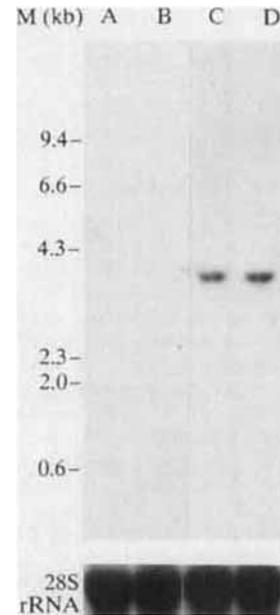

FIGURE 1 – Northern-blot analysis of human melanoma cell lines; 10 μg of total RNA were loaded in each lane. Lane A, 530; lane B, 1F6; lane C, BLM; lane D, MV3. The blot was hybridized to radiolabeled 3.5-kb human TGase2 cDNA. The MW marker was λ DNA digested with restriction enzyme Hind111. Below: control hybridization, using a 28S ribosomal probe.
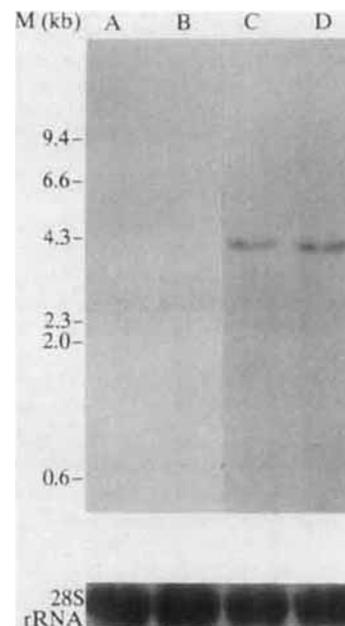

FIGURE 2 – Northern-blot analysis of melanoma xenografts in nude mice; 20 μg of total RNA were loaded in each lane. Lane A, 530; lane B, 1F6; lane C, BLM; lane D, MV3. The blot was hybridized to radiolabeled 3.5-kb human TGase2 cDNA. The MW marker was λ DNA digested with Hind111. Below: control hybridization, using a 28S ribosomal probe.

TGase enzymatic activity in human melanoma cell lines

For further characterization of the TGase2 expression, the enzymatic activity was measured in cytoplasmic extracts prepared from the human melanoma cell lines with differing
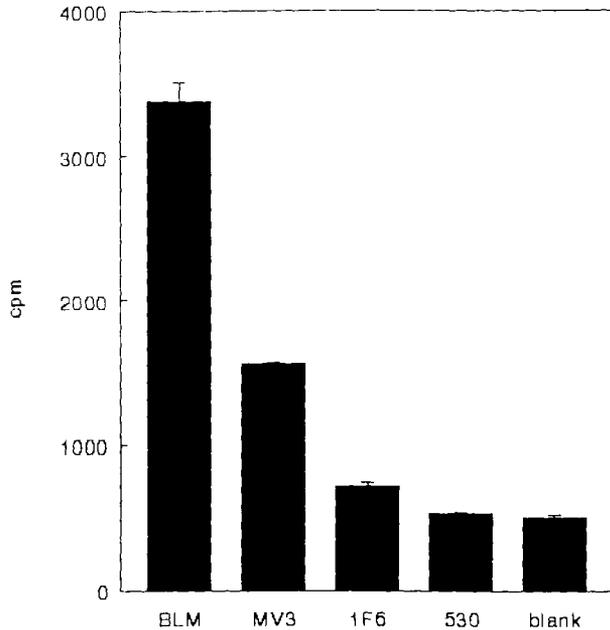
FIGURE 3 – Levels of TGase activity in the human melanoma cell lines BLM, MV3, 1F6 and 530. TGase activity was determined on the cell extracts as described in the text. The blank activity represents assays with BLM and MV3 extracts in the presence of 50 mM EDTA. Data represent the mean values ± SE from 3 experiments.

FIGURE 4 – Identification of TGase substrate(s) in several human melanoma cell lines; 30 µg extract was loaded on each lane of a 12.5% SDS-polyacrylamide gel after [^{14}C]methylamine incorporation as described in the text. Panel (a) shows the Coomassie brilliant blue staining pattern and (b) the corresponding autoradiograph. Lanes A and B, BLM; lanes C and D, MV3; lane E, 1F6; lane F, 530. Lanes B and D, [^{14}C]methylamine incorporation assay in the presence of 50 mM EDTA.

FIGURE 5 – Immunoprecipitation of TGase from several human melanoma cell lines; 2.5 × 10^6 cpm of [^{35}S]methionine-labeled cell extracts were used for each precipitation, and the immunoprecipitates were analyzed on a 10% SDS-acrylamide gel. Lanes A and B, MV3; lanes C and D, BLM; lanes E and F, 530; lanes G and H, 1F6. Lanes A, C, E, and G were only incubated with protein A-Sepharose without addition of the antibody. The arrowhead indicates the position of human TGase2.

metastatic behavior. The assay tested endogenous TGase activity acting on endogenous substrates. As shown in Figure 3, the TGase activity in the highly metastatic cell lines MV3 and BLM was significantly higher ($p \leq 0.05$ using a 2-sample t-test) than in the weakly metastatic cell lines 1F6 and 530, of which only the former showed a low level of TGase activity. This result is in agreement with the differential expression of TGase2 transcripts shown in Figure 1. Some control assays were performed in which purified guinea-pig TGase and/or caseine, a common TGase substrate, were included in the reaction mixtures (results not shown). These control assays make it clear that the observed TGase activity profile is likely to reflect the corresponding differential mRNA expression pattern and cannot be explained by significant differences in the amounts of substrate proteins or specific enzyme inhibitors.

For a preliminary identification of the TGase substrates in the human melanoma cell lines, [^{14}C]methylamine incorporation into proteins of the cytoplasmic extracts was analyzed on a 12.5% SDS-polyacrylamide gel (Fig. 4). As expected from the enzyme assays, substrate-incorporated [^{14}C]methylamine could be detected only in BLM and MV3 extracts (lanes A and C, respectively). As a consequence of its calcium dependence (Greenberg et al., 1991), the TGase activity was completely inhibited in the BLM and MV3 extracts in the presence of 10 mM EDTA (lanes B, D). The autoradiogram shows preferential labelling of some selected substrates with a few typical differences between BLM and MV3.

Immunoprecipitation of TGase2

To examine TGase2 protein expression in the human melanoma cell lines, we performed immunoprecipitations on [^{35}S]methionine-labeled cell extracts with anti-human tissue TGase2 antibodies. The immunoprecipitates obtained from the highly metastatic cell lines MV3 (lane B) and BLM (lane D), contain the expected 80-kDa TGase2 protein (Fig. 5). Trace amounts of TGase2 are detectable in the weakly
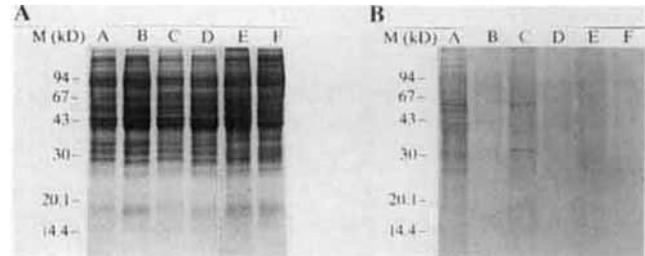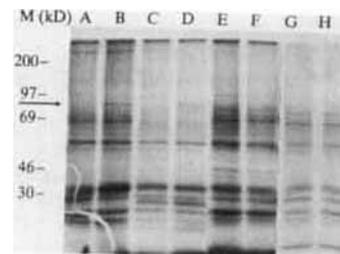
metastatic cell line 1F6 (lane H), while expression is absent in the 530 cell line (lane F). TGase2 was specifically precipitated by the affinity-purified anti-human tissue TGase2 rabbit IgG antibody, since the 80-kDa band is absent from control precipitations, in which only protein A-Sepharose was used with exclusion of the antibody (lanes A, C, E, G). In summary, all applied assays (Northern-blot analysis, enzyme activity tests, immunoprecipitations) yield corresponding results and support the conclusion that TGase2 expression is elevated in metastasizing human melanoma cell lines.

DISCUSSION

Tissue-type transglutaminase (TGase2) is a ubiquitous enzyme, mainly because of its constitutive expression in smooth muscle and endothelium of the vessel wall, while it is inducible in most other cell types (Thomazy and Fesus, 1989). The enzymatic function of TGase2 is well characterized and, among its biological functions, a participation in endocytosis (Davies et al., 1980), cellular adhesion (Gentile et al., 1992; Martinez et al., 1989) and apoptosis (Fesus et al., 1987; Alaoui et al., 1992) has been suggested. Additional experimental data are still required to characterize the suggested biological roles in detail.

Our results, as reported here, reveal a positive correlation between TGase2 expression and metastatic behavior of human melanoma cell lines. We detected elevated expression of TGase mRNA only in the highly metastatic human melanoma cell lines MV3 and BLM. The differential expression of

TGase2 mRNA is also reflected at the protein and activity levels, as shown by immunoprecipitations and enzyme activity measurements.

The correlation between TGase2 expression and neoplastic progression has been the subject of several studies dealing with animal tumor-cell lines. These studies revealed an inverse relation between metastatic behavior and expression of TGase2 (Beninati et al., 1993; Knight et al., 1990; Hand et al., 1988). It was anticipated that the presence of TGase2 would be unfavorable for tumor progression. Such an assumption would be in accordance with its suggested promoting role in processes such as apoptosis and cell adhesion. Our data, however, do not support this assumption. Therefore, prudence is required, at the moment, in assigning functional relevance to TGase2 expression in tumor progression studies without clear experimental evidence for such a role, be it beneficial or harmful.

While others have addressed the inverse relation between TGase2 expression and tumor progression (Beninati et al., 1993; Knight et al., 1990; Hand et al., 1988), it is appropriate in the context of our data to look for an explanation of TGase2 expression in metastasizing cells. Several factors have been reported to be inducers of human TGase2, including retinoic acid (Suedhoff et al., 1990), transforming growth factor-β (George et al., 1990), and interleukin-6 (Suto et al., 1993). Gain of interleukin-6 expression frequently accompanies acquisition of metastatic properties in human melanoma cells (Lu and Kerbel, 1993). In the investigated panel only the MV3 and BLM cell lines express interleukin-6 (data not shown). Alterna-

tively, if the expression of TGase2 in melanoma cells may be interpreted as a marker for apoptosis, this would mean that the highly metastatic cells can intrinsically monitor the accumulating mutations that should normally trigger this process, but somehow lack the ability to undergo programmed cell death.

What might be the functional significance of TGase2 expression in metastasizing human melanoma cells? It could indicate that a deficient apoptosis program is triggered. As another speculation, one could imagine that the enzyme might catalyze cross-linking of extracellular-matrix-derived protein fragments to plasma membrane constituents and, thus, build up a more protective shell around the cell thus increasing its chances of survival in the circulation.

Whatever the explanation, the elevated TGase2 expression observed in metastasizing melanoma cells can be used as an additional feature and experimental tool for characterizing changes occurring during neoplastic progression. It should be investigated whether the correlation between elevated TGase2 expression and the progression stage of human tumor cell lines and tumors is a more general occurrence and also whether TGase2 expression may serve diagnostic purposes. These topics will be the subject of future studies.

ACKNOWLEDGEMENTS

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