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Impairment of the metastatic activity of melanoma cells by transglutaminase-catalyzed incorporation of polyamines into laminin and Matrigel

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Summary. Previously published evidences highlighted the effect of transglutaminase (TG, EC 2.3.2.13) activation on the reduction of the in vitro adhesive and invasive behaviour of murine B16-F10 melanoma cells, as well as in vivo. Here, we investigated the influence of spermidine (SPD) incorporation by TG into basement membrane components i.e. laminin (LN) or Matrigel (MG), on the adhesion and invasion of B16-F10 melanoma cells by these TG/SPD-modified substrates. The adhesion assays showed that cell binding to the TG/SPD-modified LN was reduced by 30%, when compared to untreated LN, whereas the reduction obtained using TG/SPD-modified MG was 35%. Similarly, tumor cell invasion by the Boyden chamber system through TG/SPD modified LN or MG was respectively reduced by 45%, and by 69%. Evaluation of matrix metalloproteinase (gelatinases MMP-2 and MMP-9) activities by gel-zymography showed that MMP-2 activity was unaffected, while MMP-9 activity was reduced by about 32% using TG/SPD-modified substrate. These results strongly suggest that the observed antiinvasive effect of TG activation in the host may be ascribed to the covalent incorporation of polyamines, which led to the post-translational modification of some components of the cell basement membrane. This modification may interfere with the metastatic property of melanoma cells, affecting the proteolytic activity necessary for their migration and invasion activities.

Keywords: Melanoma cells – Invasion – Transglutaminase – Polyamines

Introduction

The metastatic process is comprised of a complex sequence of steps which require the tumor cells to be migratory, adherent to the endothelium and to the basement membrane components, and invasive (Nicolson, 1989; Fidler, 1990). The endothelium and endothelial basement membrane are believed to act as barriers to the passage of tumor cells. The past decade has witnessed substantial progress in our understanding of the molecular mechanisms of tumor cell interactions with vascular endothelium and extracellular matrix (ECM). This progress has been made possible by the identification and functional characterization of a large number of adhesion molecules involved in tumor cell-vasculature and tumor cell-basement membrane interactions (Tang and Honn, 1994).

Basement membranes are composed mostly of LN, collagen type IV, heparan sulphate proteoglycan and entactin/nidogen (Timpl et al., 1984). It is generally believed that in the basement membrane, a network of collagen IV molecules linked end to end forms the principle structural element. LN binds to the collagen IV network, to itself, to entactin, and to heparan sulphate proteoglycan, forming different structures in the basement membranes depending on the number and type of its associations with these matrix components (Martin and Timpl, 1987). LN alone conveys many of the biological effects of basement membrane and appears to enhance the malignant phenotype. It promotes tumor cell adhesion, migration, growth, and resistance to cytotoxic drugs (Barsky et al., 1984; Terranova et al., 1984; Liotta et al., 1986), as well as the activity of collagenase IV, enzyme involved in the invasion process (Kleinman et al., 1991). Moreover, LN receptors has been found to correlate positively with the tumorigenicity of cells (Wewer et al., 1986) and with the stage of malignancy of human tumors (Yow

et al., 1988; Berno et al., 2005). Thus, tumor cells interact with multiple proteins but particularly with LN in basement membrane, and the level of LN receptors on some tumors may be used to predict their malignant behaviour.

TGs are a family of Ca^{2+}-dependent enzymes that catalyze the incorporation of primary amines into protein-bound glutamine residues via an acyl-transfer reaction (Folk, 1980). A widespread form of the enzyme has been identified in a number of mammalian cell types, in both non-differentiated and differentiated cells (Maddox and Haddox, 1985), as well as in tumor cell types (Dadabay and Pike, 1989). The biological role of tissue transglutaminase (tTG) is not well understood, but evidence has suggested its involvement in a number of activities, including cell growth and differentiation (Birckbichler and Patterson, 1978), cytoskeletal stabilization (Maccioni and Arechaga, 1986), and cell-matrix interactions (Aeschlimann and Thomazy, 2000). Many evidences demonstrate that the increase of TG activity in melanoma cells reduces their malignant behaviour (Beninati et al., 1993; Lentini et al., 1997, 1998, 2007), and that its augmented expression in the host parallels the inhibition of invasion and the growth of a mammary adenocarcinoma implanted in rat (Haroon et al., 1999). Since the ability of metastatic cells to invade the target organs involves the interaction with the basement membrane and its proteolytic digestion, the aim of this work is to investigate whether the covalent modification of basement membrane proteins by TG may interfere with the adhesiveness and invasiveness of tumor cells. Interestingly, LN, collagen and various types of extracellular matrix proteins represent good substrates for TG catalysis (Mosher, 1984; Aeschlimann and Paulsson, 1991; Aeschlimann et al., 1992; Beninati et al., 1994; Kaartineen et al., 1999; Jones et al., 2006), and this fact might support the involvement of the enzyme in the migration and adhesion processes occurring during the metastatic spread of cancer cells. Here, we have investigated the effect of the TG-catalyzed post-translational incorporation of SPD into basement membrane substrates on the adhesion and invasion capacity of murine B16-F10 melanoma cells. As a main component of the basement membrane, we used LN as a known TG substrate, which represent the main component of basement membranes, and MG, a tumor-derived mixture of LN, collagen IV, heparan sulfate and entactin, which has been biochemically and ultrastructurally related to a basement membrane (Grant et al., 1985; Kleinman et al., 1986).

Materials and methods

Materials

All chemicals were Sigma-reagent grade and were used without further purification. Terminal methylene-[^{3}H]-spermidine dihydrochloride (18 Ci/mmol) was obtained from NEN (Boston, MA, USA). Guinea pig liver TG (GPL-TG), mouse LN, spermidine (SPD) and bovine serum albumin (BSA) were supplied from Sigma (St. Louis, MO, USA). MG was purchased from Collaborative Medical Products (Bedford, MA, USA). Fetal calf serum (FCS), Dulbecco's minimum essential medium (D-MEM) and ethylene diamine tetraacetic acid (EDTA) were from Gibco Laboratories (Grand Island, NY, USA). All other chemicals were provided by Merck (Darmstadt, Germany).

Incorporation of [^{3}H]-SPD or unlabelled SPD into LN and MG

The incorporation of [^{3}H]-SPD by GPL-TG into LN or MG was performed according to Chung and Folk (1972). Substrates (1 mg) were incubated with GPL-TG in the presence of SPD and Ca^{++}. Control experiments were performed using EDTA as TG inhibitor. The same procedure was followed in order to prepare the substrates conjugated with non-radioactive SPD. The concentration of SPD covalently incorporated in LN was 41.0 ± 2.3 (S.D.) pmoles SPD/mg protein and in MG was 60.0 ± 3.2 (S.D.) pmoles SPD/mg protein.

Cell cultures

B16-F10 highly metastatic murine melanoma cells (obtained from I. J. Fidler, University of Texas, M.D. Anderson Cancer Center, Houston, TX, USA) were propagated under standard culture conditions (Fidler, 1973). Cultures were found to be free from *Mycoplasma* species using the Hoechst staining procedure and the invasive potential was assessed in an in vitro invasion assay, as previously described (Albini et al., 1987). 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).

Adhesion assays

The adhesion assays were performed on 24-wells plates coated with untreated or TG/SPD-modified LN or MG (1, 5, 10, 50 and 100 µg, in D-MEM), and dried overnight at 37 °C. Unbound surfaces were blocked with 3% BSA in D-MEM (500 µl/well) for 30 min at 37 °C, and then aspirated prior to the addition of cells. Cells were harvested with EDTA and resuspended in 0.02% BSA in D-MEM. 8×10^5 cells/well were incubated for 1 h at 37 °C (5% CO_2). After extensive washings with PBS (pH 7.4), attached cells were detached with trypsin/EDTA for 10 min at room temperature and counted with a Coulter counter. Each condition was tested in triplicate and the experiments were repeated twice.

Invasion assays

Invasion assays were carried out in Boyden chambers as previously described (Albini et al., 1987). Policarbonate polyvinylpyrrolidone-free membranes (8 µm pore size, Neuroprobe, Cabin John, MD, USA) were coated with 50 µl untreated or TG/SPD-treated LN or MG solutions (1 mg/ml). As an adjunctive control, free SPD (100 mM) was added to the untreated substrates. As chemoattractant, NIH3T3 fibroblast-conditioned medium (obtained after overnight incubation of subconfluent cultures in serum-free D-MEM with 5% ascorbate) was used. Cells harvested with EDTA were counted, resuspended in D-MEM containing 0.3% BSA, and incubated at 2×10^5cells/chamber for 5 h at 37 °C. Invasive cells were fixed in 100% ethanol and stained with haematoxylin and eosin. Each

condition was tested in triplicate. The cell density was measured using an Optimax V HR morphometric image analyser (Analytical Measuring Systems, Essex, UK).

Gel-zymography

Gel-zymographic analyses were performed as previously described (Chang and Szabo, 2000). To examine metalloproteinases MMP-2 and MMP-9 activities, conditioned medium from B16-F10 melanoma cells cultured without serum was concentrated 10-fold. Aliquots were normalized for cell number and run on 10% zymogram gels containing 1% MG (control) or TG/SPD-modified MG (60.0 ± 3.2 pmoles SPD/mg of MG). The integrated optical density (IOD) of the gelatinase bands were measured with Image Pro-Plus software (Media Cybernetics).

Statistical analysis

Data were analyzed using the Student's unpaired *t*-test. Differences were considered to be significant at p values < 0.001.

Results

Incorporation of [^{3}H]-SPD and unlabelled SPD into LN and MG

A representative experiment showing the TG-catalyzed incorporation of [^{3}H]-SPD into LN (a) or MG (b) is shown in Fig. 1. The radioactivity increases with time of incubation, reaching a plateau in both cases after 80 min of incubation. The amount of radioactivity found in the EDTA-containing samples (TG inhibitor) is significantly different from the Ca^{++}-containing samples ($p < 0.001$).

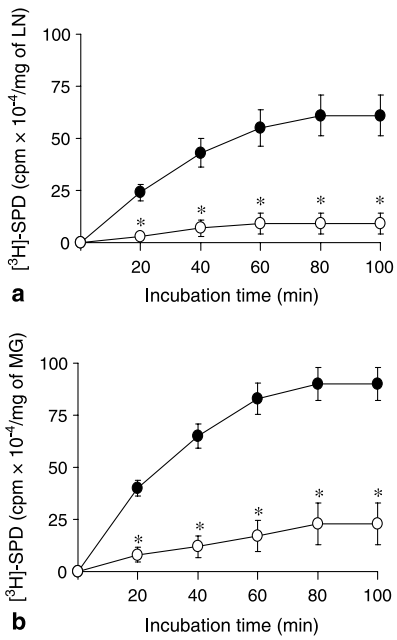

Fig. 1. GPL-TG-catalyed incorporation of [^{3}H]-SPD into LN (**a**) or MG (**b**). Data are expressed as the mean ± SD of three different determinations. ● Ca^{++}-containing sample; ○ EDTA-containing sample; *highly significant versus control ($p < 0.001$)

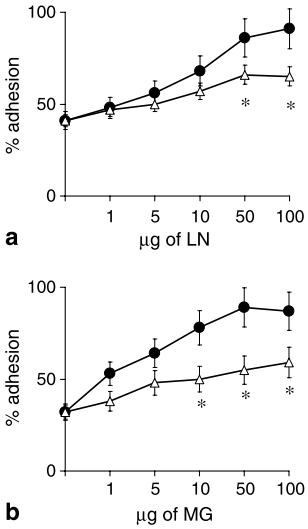

Fig. 2. Effect of TG/SPD-mediated modification of LN (**a**) or MG (**b**) on B16-F10 melanoma cells in vitro adhesion. Data represent the mean ± SD of three different determinations. ● Control substrate; △ TG/SPD-modified substrate; *highly significant versus control ($p < 0.001$)

Adhesion experiments

Figure 2 shows the adhesion of B16-F10 cells over untreated or TG/SPD-treated LN (Fig. 2a) or MG (Fig. 2b), respectively. In both experiments, the number of adherent cells increases with the concentration of the substrate used. The adhesion to the TG/SPD-modified LN lowers

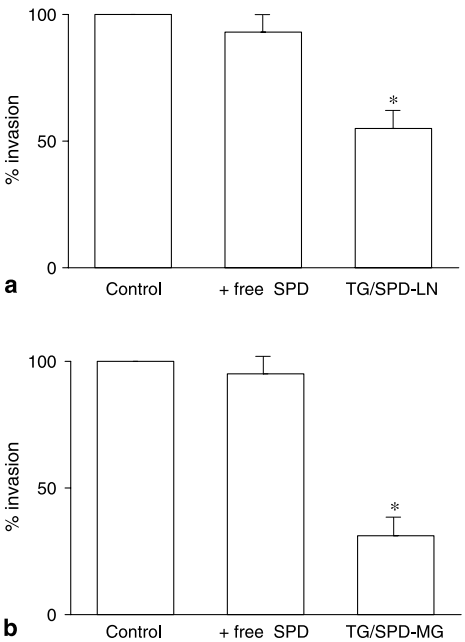

Fig. 3. Effect of TG/SPD-mediated modification of LN (**a**) or MG (**b**) on B16-F10 melanoma cells in vitro invasion. Data represent the mean ± SD of three different determinations. *Highly significant versus control ($p < 0.001$)

only at the higher concentrations of substrate (from 17% decrease for 10 μg, to 30% decrease for 100 μg, $p < 0.001$), whereas the adhesion to modified MG decreases for all concentrations used (27% decrease in the range 1–5 μg, 35% decrease in the range 10–100 μg, $p < 0.001$).

Invasion experiments

The in vitro invasion of B16-F10 melanoma cells was evaluated by coating filters with untreated or TG/SPD-modified LN or MG. Figure 3a shows that the invasive capability of B16-F10 cells was reduced by 45% ($p < 0.001$), with respect to the control, using modified LN as substrate bareer, whereas the reduction was 69% ($p < 0.001$) using modified MG as filter coating (Fig. 3b). The invasion experiments performed using the untreated substrates added with free SPD (Fig. 3a and b) did not show any difference with the control.

Activity of matrix metalloproteinases against a TG/SPD-modified basement membrane-like substrate

Figure 4 shows the gel zymography obtained loading the conditioned media from B16-F10 melanoma cells in MG-containing (a) or TG/SPD-modified MG-containing (b) gels. The evaluation of the integrated optical density (IOD) of the 72 kDa metalloproteinase-2 (MMP-2) and the 92 kDa metalloproteinase-9 (MMP-9) shows that MMP-9 activity was reduced, with respect to the control, by about 32% ($p < 0.001$) in the gel containing TG/SPD-modified MG, whereas MMP-2 activity seems to be unaffected.

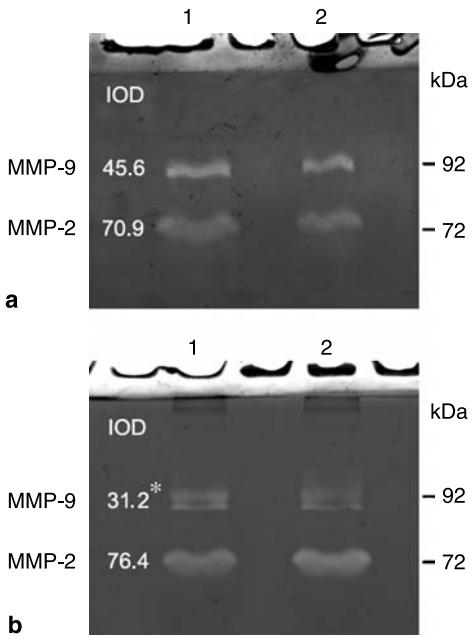

Fig. 4. Representative gel zymography of conditioned medium from B16-F10 melanoma cells. The activities of 72 kDa MMP-2 and 92 kDa MMP-9 were determined both in control MG- (**a**) and TG/SPD-modified MG- (**b**) containing gels, and expressed as integrated optical density (IOD) of the electrophoretic bands. Lanes *1* and *2* were loaded with the same sample. *Highly significant versus control ($p < 0.001$)

Discussion

Interactions of cells with the surrounding ECM are critical for many key aspects in cell behaviour, including cell adhesion, growth, migration, differentiation, programmed cell death and ECM assembly. In turn, these cellular processes are involved in embryonic development and tissue formation, wound healing and tissue repair, as well as tumor growth and metastasis. Several major classes of adhesion receptors present on various cell types include integrins, selectins, cell adhesion molecules of immunoglobulin superfamily (CAMs) and heparan sulphate proteoglycans (HSPGs), which together define the specific patterns of cellular interactions with ECM glycoproteins such as fibronectin (FN), collagens, laminins as well matrix proteoglycans (Zemskov et al., 2006).

Recently it was reported that integrins are associated with tissue TG (tTG) in a number of different cell type via binding to the extracellular domains of the β1 and β3 integrin subunits. The formation of stable non-covalent integrin-tTG complexes does not involve the transamidating activity of tTG and integrins do not appear to serve as enzymatic substrates of tTG or other TGs (Akimov et al., 2000).

In the present study, we investigated the in vitro behaviour of adhesion, migration and invasion of malignant murine melanoma cells in culture. Adhesiveness of B16-F10 melanoma cells to cellular basement membrane-like proteins (LN or MG) was reduced when the proteins were modified by SPD through the catalytic action of tTG. In order to explain this finding, it is relevant to consider the cooperation between the adhesive and protein-cross-linking function of tTG on the cell surface and in the ECM. This collaboration is based on the fact that most integrin ligands in the ECM (FN, LN, collagens, osteopontin, vitronectin, etc.) serve as enzymatic substrates for cell surface tTG (Bowness et al., 1987; Sane et al., 1988; Aeschlimann and Paulsson, 1991; Beninati et al., 1994). The major enzymatic mechanism of stabilizing the ECM structure involves multimerization of protein via covalent cross-linking by TG, making the cross-linked ECM

polymer resistant to proteolytic degradation as well as mechanical other stresses. Modification of tTG substrates by polyamines may impair the possibility of ECM cross-linking, modifying cellular responses to ECM ligands. Probably, these modifications are responsible of the observed adhesive behaviour of B16-F10 melanoma cells in our experiments.

Results demonstrated that the tumor cells line B16-F10, are able to invade through LN- or MG-coated filters in a Boyden Chamber system, but this ability was influenced by a covalent modification of the two basement membrane-components by a tTG-catalyzed incorporation of SPD. These in vitro observations provide some insights into the biological behaviour of tumor cells, when the activity of the host TG is increased (Lentini et al., 1997), leading to ECM alterations (Mangala et al., 2005) or incorporation of polyamines into suitable components of basement membranes.

B16-F10 cells were able to penetrate through the polyamine-modified LN- or MG-coated filters, but as the amount of protein substrate added to the filter increased, their ability to penetrate was reduced. It is well known that tumor cells secrete proteolytic enzymes, which possess specificity for basement membranes proteins (metalloproteinases, collagenases, etc). Therefore, it is not surprising for melanoma cells in culture to possess some ability to penetrate the LN- or MG-coated filters.

It is of interest to consider that, in addition to the interaction with integrins, tTG may associate with other proteins on the cell surface. A novel binding partner for tTG is a matrix metalloproteinases MMP-2 (Belkin et al., 2004). This finding indicates the existence of a cross-talk between matrix metalloproteinases involved in ECM degradation and tTG, which promotes cell-matrix adhesion and stabilizes the ECM. The interplay between tTG and matrix metalloproteinases is likely important for dynamic regulation of cell-matrix adhesion and migration of tumor cell in vivo.

The issue which remained in the present study was to identify the mechanism of the influence of tTG-catalyzed polyamine modification of LN or MG on the invasive property of the tumor cell line under investigation. The results obtained by the zymographic analysis of the activity of the metalloproteinases secreted by B16-F10 cells, indicated that there is a distinct difference in the affinity of gelatinase MMP-9 against the natural and the polyamine-modified basement membrane-like protein substrates. This finding assumes a particular interest, considering that MMP-9 has been correlated with rapid progression in patient with metastatic melanoma (Turpeenniemi-Hujanen, 2005).

In summary, results of the present study demonstrated that cellular behaviour in the Boyden Chamber migration and invasion assay could be affected by the presence of covalently linked polyamines to the proteins component of basement membranes, likely by the decreased affinity of these substrated for the gelatinases secreted by melanoma cells. This possibility is of interest considering that the passage of circulating tumor cell across the vascular wall is an important step in cancer invasion and metastasis. The ability of tumor cell to adhere to the endothelial cells and to the basement membrane may be crucial in the metastatic process and may dramatically alter the clinical prognosis and outcome for patients with malignant cancers.

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