MOLECULAR CANCER

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Timp1 interacts with beta-1 integrin and CD63 along melanoma genesis and confers *anoikis* resistance by activating PI3-K signaling pathway independently of Akt phosphorylation

Mariana Toricelli[1], Fabiana HM Melo[1,2], Giovani B Peres[3], Débora CP Silva[4] and Miriam G Jasiulionis[1*]

Background: *Anoikis* resistance is one of the abilities acquired along tumor progression. This characteristic is associated with metastasis development, since tumorigenic cells must survive independently of cell-matrix interactions in this process. In our laboratory, it was developed a murine melanocyte malignant transformation model associated with a sustained stressful condition. After subjecting melan-a melanocytes to 1, 2, 3 and 4 cycles of anchorage impediment, *anoikis* resistant cells were established and named 1C, 2C, 3C and 4C, respectively. These cells showed altered morphology and PMA independent cell growth, but were not tumorigenic, corresponding to pre-malignant cells. After limiting dilution of 4C pre-malignant cells, melanoma cell lines with different characteristics were obtained. Previous data from our group showed that increased Timp1 expression correlated with *anoikis*-resistant phenotype. Timp1 was shown to confer anchorage-independent growth capability to melan-a melanocytes and render melanoma cells more aggressive when injected into mice. However, the mechanisms involved in *anoikis* regulation by Timp1 in tumorigenic cells are not clear yet.

Methods: The β1-integrin and Timp1 expression were evaluated by Western blotting and CD63 protein expression by flow cytometry using specific antibodies. To analyze the interaction among Timp1, CD63 and β1-integrin, immunoprecipitation assays were performed, *anoikis* resistance capability was evaluated in the presence or not of the PI3-K inhibitors, Wortmannin and LY294002. Relative expression of *TIMP1* and *CD63* in human metastatic melanoma cells was analyzed by real time PCR.

Results: Differential association among Timp1, CD63 and β1-integrins was observed in melan-a melanocytes, 4C pre-malignant melanocytes and 4C11- and 4C11+ melanoma cells. Timp1 present in conditioned medium of melanoma cells rendered melan-a melanocytes *anoikis*-resistant through PI3-K signaling pathway independently of Akt activation. In human melanoma cell lines, in which TIMP1 and beta-1 integrin were also found to be interacting, *TIMP1* and *CD63* levels together was shown to correlate significantly with colony formation capacity.

Conclusions: Our results show that Timp1 is assembled in a supramolecular complex containing CD63 and β1-integrins along melanoma genesis and confers *anoikis* resistance by activating PI3-K signaling pathway, independently of Akt phosphorylation. In addition, our data point *TIMP1*, mainly together with *CD63*, as a potential biomarker of melanoma.

Keywords: *Timp1*, Beta1-integrin, CD63, PI3-K pathway, *Anoikis*, Melanoma, Malignant transformation

* Correspondence: mjasiulionis@gmail.com
[1]Pharmacology Department, Universidade Federal de São Paulo, São Paulo, Brazil
Full list of author information is available at the end of the article

Introduction

Cutaneous melanoma originates from malignant transformation of melanocytes. As in the case of most cancers, it is believed that genetic and environmental factors contribute to development of melanoma. Although melanoma is the less frequent skin cancer (about 4%), it is responsible for most deaths (approximately 80%) due its high incidence of metastases [1,2]. Cell-extracellular matrix interactions are a key factor for melanocyte homeostasis and disruption of such interactions has adverse effects on cell survival, driving a specific type of apoptosis known as *anoikis*. This kind of apoptosis following loss of cell anchorage is important for development, tissue homeostasis and several diseases, including cancer [3]. *Anoikis* has a particular importance in tumor progression, since the acquisition of an *anoikis*-resistant phenotype is one of the critical steps acquired during malignant transformation [4]. In animal models, *anoikis*-resistant tumor cells are associated with high incidence of metastatic lesions and increased cell survival in blood [5-8]. Furthermore, this property observed in these cells when cultured *in vitro* can be correlated to its oncogenic potential found *in vivo* [9].

A previous work of our group showed that the acquisition of *anoikis*-resistant phenotype is associated with melanocyte malignant transformation [10]. Oba-Shinjo and colleagues (2006) demonstrated that sequential cycles of anchorage blockade resulted in malignant transformation of melan-a murine melanocytes [10]. As expected for a non-tumorigenic immortalized cell line, most melan-a cells underwent *anoikis* when maintained in suspension. However, small spheroids were observed after maintaining melan-a cells in suspension for 96 hours. Melan-a cells resistant to *anoikis* were cultured in adherent conditions and subjected to further deadhesion cycles. Cell lines were established after subjecting melan-a melanocytes to 1, 2, 3 and 4 cycles of anchorage blockade (1C, 2C, 3C and 4C cell lines, respectively). These cell lines were non-tumorigenic, but showed morphological changes, increased *anoikis*-resistant phenotype and alterations in the expression of different molecules [10], being considered pre-malignant cells. Pre-malignant 4C melanocytes were subjected to a new cycle of anchorage blockade and the spheroids formed were submitted to a limiting dilution. All clones, randomly selected, were tumorigenic when subcutaneously inoculated into syngeneic mice. In this way, different melanoma cell lines (i.e. 4C11- and 4C11+) were established and showed differences in pigmentation, aggregation, *in vitro* and *in vivo* proliferation and ability to metastasize [10].

TIMP1 is a member of the family of matrix metalloproteinase (MMP) inhibitors that is composed of four members (TIMP1, TIMP2, TIMP3 and TIMP4). As the name suggests, the main function of TIMP1 is to inhibit extracellular matrix degradation mediated by MMPs. However, TIMP1 may interact with other proteins and regulate biological processes such as cell growth, apoptosis and differentiation, independently of its metalloproteinase inhibitory activity [11-15]. In this context, CD63, a member of the tetraspanin family, was first identified as an antigen associated with early stages of human melanoma and as a binding partner of TIMP1 on the cell surface [15]. Jung and coworkers [15] showed a correlation between the expression of TIMP1 and the level of active β1-integrin on the surface of epithelial breast cells, independent of cell adhesion, and showed interaction among TIMP1, CD63 and β1-integrin. Inhibition of CD63 expression was able to effectively reduce TIMP1 binding on the cell surface and its co-localization with β1-integrins. Besides that, β1-integrins activation, signaling survival activation and inhibition of apoptosis mediated by TIMP1 was abrogated. The mammals' integrin family contains 18 α-subunits and eight β-subunits that form 24 distinct receptors with specific tissue distribution that appear to have specific and non-redundant functions as shown by their specificity for ECM ligands and knockout mouse phenotypes [16]. The primary function of integrin family is to mediate cell-cell and cell-matrix adhesion. Furthermore, the binding of ECM components to integrins leads to the recruitment of numerous adaptor and signaling proteins to the cytoplasmic tails of the integrin β-subunits, forming adhesion protein complexes that initiate signaling cascades promoting cell polarity, motility, differentiation, proliferation and survival [16]. β1-integrins are expressed in a wide variety of tissues and different cell types throughout the body. They are critical in the induction and maintenance of cell differentiation and are involved in various physiological functions and in tissue homeostasis [16].

Previously, we reported increased *Timp1* expression along melanoma genesis and a tight correlation between *Timp1* expression and its promoter methylation during melanocytes malignant transformation [17]. It was demonstrated that Timp1 confers resistance to *anoikis*, since melanocytes overexpressing Timp1 become able to resist to *anoikis* and form colonies in soft-agar. Moreover, melanoma cells overexpressing Timp1 acquire increased capacity to grow and metastasize *in vivo* [17]. However, the signaling pathway induced by Timp1 to protect melanoma cells from apoptosis is still unknown.

The phosphoinositide 3-kinase (PI3K) signaling pathway activation regulates fundamental cellular func- tions such as transcription, growth, differentiation and survival, mostly through AKT phosphorylation [18]. PI3K/AKT signaling is associated with the disruption in the balance between cell proliferation and apop- tosis, and has been related with the development of

diseases such as cancer, autoimmunity and diabetes mellitus [18].

In this work, we have shown for the first time the assembly of a supramolecular complex containing Timp1, CD63 and β1-integrins at the cell surface in melanoma cells, and its involvement in the acquisition of an *anoikis*-resistant phenotype through PI3K signaling pathway independently of Akt activation. In addition, our data points TIMP1 as a biomarker of human melanoma.

Material and methods

Cell culture

The non-tumorigenic melan-a melanocyte lineage [19] was cultured at 37°C in humidified 95% air-5% CO_2 in RPMI pH 6.9 supplemented with 5% fetal bovine serum (Invitrogen, Scotland, UK), 200 nM 12-phorbol-13-myristate acetate (PMA; Calbiochem, Darmstadt, Germany), 100 U/ml penicillin, and 100 U/ml streptomycin (Invitrogen, Grand Island, NY). PMA activates protein kinase C, and is required for melanocytes to survival and proliferate in culture [19]. Pre-malignant 4C melanocyte lineage, non-metastatic 4C11- and metastatic 4C11+ melanoma cell lines were cultured as melan-a cells, but in the absence of PMA, since they lost the requirement of this factor to grow. Stably Timp1-overexpressing melan-a (MaT1S) and the control MaGFP were cultured in the same conditions described above in the presence of PMA. The plasmid construction and transfection was previously described [17]. Primary human melanocytes MP#2, kindly provided by Dr. Silvya Stuchi Maria-Engler (Faculdade de Ciências Farmacêuticas, Universidade de São Paulo), was cultured at 37°C in humidified 95% air-5 % CO_2 in Cascade growth medium 254 (Gibco, Grand Island, NY) supplemented with 200 μM $CaCl_2$ and 1% HGMS (human growth melanocyte supplement). The patient-derived metastatic melanoma cells Mel2, Mel3, Mel4, Mel11, Mel14, Mel21, Mel25, Mel28 and Mel33 kindly provided by Dr. Débora C.P. Silva (Ludwig Institute for Cancer Research, São Paulo), were cultured at 37°C in humidified 95% air-5% CO_2 in RPMI pH 7.2 supplemented with 15% fetal bovine serum (Invitrogen, Scotland, UK), 1 mM sodium piruvate, 2 mM L-glutamine and antibiotics (Gibco, Grand Island, NY).

mRNA expression analysis

RNA was isolated from cell monolayers (MP#2, Mel2, Mel3, Mel11, Mel14, Mel21, Mel25, Mel33, Melan-a, 4C, 4C11- and 4C11+) using TRIzol® (Invitrogen, Carlsbad, CA). cDNA was prepared from 1 μg of RNA using random hexamer primers and OligodT (Superscript III first-strand synthesis system for RT-PCR, Invitrogen). For *TIMP1* and *CD63* mRNA expression analysis, quantitative real-time PCR were performed using a Corbett Rotor-Gene 6000 detection system® with a Fast Rotor-Gene SYBR Green PCR Master Mix® (Qiagen, Dusseldorf, Germany). Specific primers were used as follows: human *TIMP1* (sense: 5' ATG TTA CTG TGG GCT GTG G 3', antisense: 5' CGA CAA AAG CAA TTC CAA GGG 3'), human *CD63* (sense: 5' CCC TTG GAA TTG CTT TTG TCG 3', antisense: 5' CGT AGC CAC TTC TGA TAC TCT TC 3'), human *GAPDH* (sense: 5' GTC TTC CCC TCC ATC GTG 3', antisense: 5' GTA CTT CAG GGT GAG GAT GC 3'), mouse *timp1* (sense: 5' GCT AAA AGG ATT CAA GGC 3', antisense: 5' GCA CAA GCC TAG ATT CCG 3'), and mouse *gapdh* (sense: 5' CCA TGG AGA AGG CTG GGG 3' antisense: 5' CAA AGT GTC ATG GAT GAC A 5').

Western Blotting

Subconfluent cell cultures were trypsinized, washed with PBS and membrane-enriched protein extracts were prepared using cold lysis buffer (1% Triton X-100 in 150 mM NaCl and 50 mM Tris pH 7.4, containing 5 mM EDTA, CHAPS 1%, 2 g/mL aprotinin and 1 mM PMSF), kept for 15 minutes on ice, followed by centrifugation at 10.000 rpm for 15 minutes at 4°C. Alternatively, MaT1S and MaGFP cells submitted for 1 h (D1h), 3 h (D3h), 5 h (D5h) and 24 h (D24h) were washed and cytoplasmic protein extracts were carried out using cold lysis buffer (0.5% NP40 in 100 mM NaCl and 50 mM Tris pH7.4, containing 50 mM NaF, 1 mM $NaVO_4$, 2 g/mL aprotinin and 1 mM PMSF), kept for 15 minutes on ice, followed by centrifugation at 10.000 rpm for 15 minutes at 4°C. The supernatant was collected and protein concentration was measured by Bio-Rad protein assay dye reagent concentrate (Bio-Rad, Hercules, CA). Equivalent amounts of protein (50 μg) were denaturated in SDS sample buffer (240 mM Tris–HCl pH 6.8, 0.8% SDS, 200 mM beta- mercaptoethanol, 40% glycerol and 0.02% bromophenol blue) for 5 minutes, then separated by electrophoresis in SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Amersham, Piscataway, NJ). After protein transfer, the membranes were blocked with 5% non-fat dry milk in PBS (10mM phosphate buffer pH 7.2, 150 mM NaCl), incubated with the indicated antibodies: goat polyclonal anti-Timp1 (R&D), rabbit polyclonal anti-β1-integrin (Calbiochem), rabbit polyclonal anti-CD63 (Santa Cruz), rabbit polyclonal anti-phospho Akt (Cell Signaling) and rabbit polyclonal Akt (Cell Signaling) overnight at 4°C, and the signal was detected using horseradish peroxidase–conjugated anti–immunoglobulin G antibody (KPL; Gaithersburg, MD) followed by development using chemiluminescence substrate (SuperSignal West Pico Chemiluminescent Substrate; Pierce Chemical, Rockford, IL).

Metalloprotease activity

Gelatinase activity was determined by zymography, as previously described [20,21]. In brief, 48 hours conditioned media (10 μg) were submitted to 10% polyacrilamide gel electrophoresis with 1% gelatin as substrate (30 mA per gel, in ice bath). After run, gels were washed four times in 2% Triton X-100, 5 min each, in order to remove SDS and renaturate enzymes. Gels were then incubated overnight at 37°C in 50 mM Tris–HCl buffer pH 8.2, containing 5 mM $CaCl_2$ and 0.5 μM $ZnCl_2$ (incubation buffer). Gels were stained with 0.5% Coomasie Brilliant Blue R-250 in 30% methanol, 10% acetic acid for 30 min, and then properly destained until clear (degraded) bands could be seen. Zymograms were scanned with Epson Expression 1680 flatbed scanner (Epson America, Inc., Long Beach, CA, USA), and densitometric analysis was performed with TotalLab Quant v11 (TotalLab Ltd., Newcastle, UK). Experiments were always performed in quadruplicates.

Colony formation assay

MaGFP, MaT1S, MP#2, Mel2, Mel3, Mel4, Mel11, Mel 25, Mel33 cells (1×10^2) were cultured in adherent conditions in complete medium on 6-well plates to allow colony formation. After seven days, colonies were washed with PBS, fixed in 3.7% (v/v) formaldehyde for 15 minutes, stained with 1% Toluidine blue 1% Borax for 5 minutes and washed with water. For quantification of survival cells, the staining was dissolved in 1% SDS and the absorbance at 570 nm was evaluated using an ELISA microplate reader.

Flow cytometry analysis

Cultured cells were harvested with trypsin. After trypsin inactivation, cells were washed with PBS containing 1% BSA and incubated with anti-CD63 (Santa Cruz) diluted in 1% BSA in PBS for 1 hour under agitation at 4°C. After incubation, cells were washed with PBS and incubated with secondary antibody anti-IgG Alexa-488 (Molecular Probes) for 45 minutes under agitation at room temperature. After three washes with PBS containing 0.1% BSA, cells were analyzed in a flow cytometer (FACScalibur, Becton Dickinson).

Co-immunoprecipitation assays

Extracts from murine melan-a, 4C, 4C11- and 4C11+ cell lines and human MP#2, Mel2, Mel3, Mel14 cell lines were pre-cleared with 50 μl of G protein-coupled to agarose beads (Gibco BRL, Gaitherburg, MD) for 2 hours at 4°C under agitation. The suspension was centrifuged at 10.000 rpm for 15 minutes at 4°C and the collected supernatant was incubated overnight at 4°C with specific antibodies against CD63 (Santa Cruz), β1-integrin (Calbiochem) and TIMP1 (Santa Cruz). The suspension was incubated with G protein-agarose for 4h at 4°C under agitation and centrifuged at 6000 rpm for 10 seconds at 4°C. The beads were collected and washed three times for 10 minutes at 4°C with wash buffer A (10 mM Tris pH 8.0, 150 mM NaCl, 0.1% Triton X-100) and two times with wash buffer B (10 mMTris pH 6.8). Beads were added to 60 μL of reducing sample buffer, which were boiled for 5 min, separated by electrophoresis on polyacrylamide-SDS 10% and 15%, transferred to PVDF membrane and incubated with antibodies of interest.

Conditioned medium

Melan-a and 4C11+ cell lines (1×10^6) were maintained in RMPI medium pH 6.9 with 0.5% fetal bovine serum for 48 hours. After 48 hours, the supernatant was collected and centrifuged at 2000 rpm for 3 minutes and used in cell survival experiments.

Anoikis resistance assay

This assay was performed in two ways. First, to evaluate the relative number of surviving cells (adherent and suspended cells) after deadhesion, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed. For this, adherent melan-a cells were harvested by mild trypsin treatment and 1×10^5 cells were cultured per mL on 1% (w/v) agarose-coated plates for 96 hours (D96h) in the absence or presence of melan-a or 4C11+ conditioned medium and in the absence or presence of 20 μL of Timp1-neutralizing antibody (R&D) in fresh medium containing 5% (v/v) FBS. After 96 h in suspension, cells were collected, transferred to 96-well plate and incubated with MTT. To ensure that all viable cells would be analyzed, the plate was centrifuged, cells were lysed with isopropanol and the absorbance at 620 nm was recorded in each well using an ELISA microplate reader. This experiment was performed in biological triplicate. Statistical analysis were made using Student's T test for unpaired samples. Second, to evaluate the relative number of cells able to form colonies after submitted to anchorage impediment, melan-a, 4C11-, 4C11+, MaT1S and MaGFP cells were cultured in complete medium for 24 hours on 6-well plates. These cells were harvested by mild trypsin treatment and 2.5×10^3 cells per mL were cultured on 1% (w/v) agarose-coated plates for 96 hours (D96h) in the absence or presence of melan-a or 4C11+ conditioned medium, Timp1 neutralizing antibody (R&D), 0.5 μM Wortmannin, a PI3-K inhibitor, (Calbiochem, Darmstadt, Germany) or 2.5 μM LY294002, a PI3-K specific inhibitor (Sigma) in fresh medium containing 0.5% (v/v) FBS at 37°C in 5% CO_2. The cells were collected by centrifugation, seeded on 60 mm-dishes and grown for five days to allow colony formation. Colonies were washed with PBS, fixed in 3.7% (v/v) formaldehyde

for 15 minutes, stained with 1% Toluidine blue for 5 minutes and washed with water. For quantification of survival cells, the staining was lysed in 1% SDS and absorbance at 570 nm was evaluated using an ELISA microplate reader.

Statistical analysis

All tests were conducted in biological triplicate. The results were organized into a database using the statistical program GraphPad Prism 5 (GraphPad Software, Inc. - California, USA). The level of significance utilized was $p<0.05$. The statistical tests performed were: Student's T test for unpaired samples, One-way ANOVA followed by Tukey's multiple comparison tests and Spearman's Rank Correlation.

Results

Increased levels of membrane-bound and secreted Timp1 in cells representing melanoma progression

Previous data from our laboratory have shown significant and progressive increase in the expression of *timp1* in cells representing different phases of melanoma progression. This increase was accompanied by *anoikis* resistance and more aggressive phenotype *in vivo* [17]. In agreement with our previous data, a progressive increase of Timp1 mRNA expression was found in 4C11- and 4C11+ melanoma cell lines (Figure 1A), as described for other melanoma cell lines from our model [17]. To analyze the Timp1 protein expression and especially its association with cell surface, membrane-enriched extracts of melan-a, 4C, 4C11- and 4C11+ cell lines were separated by SDS/PAGE. Timp1 protein expression was

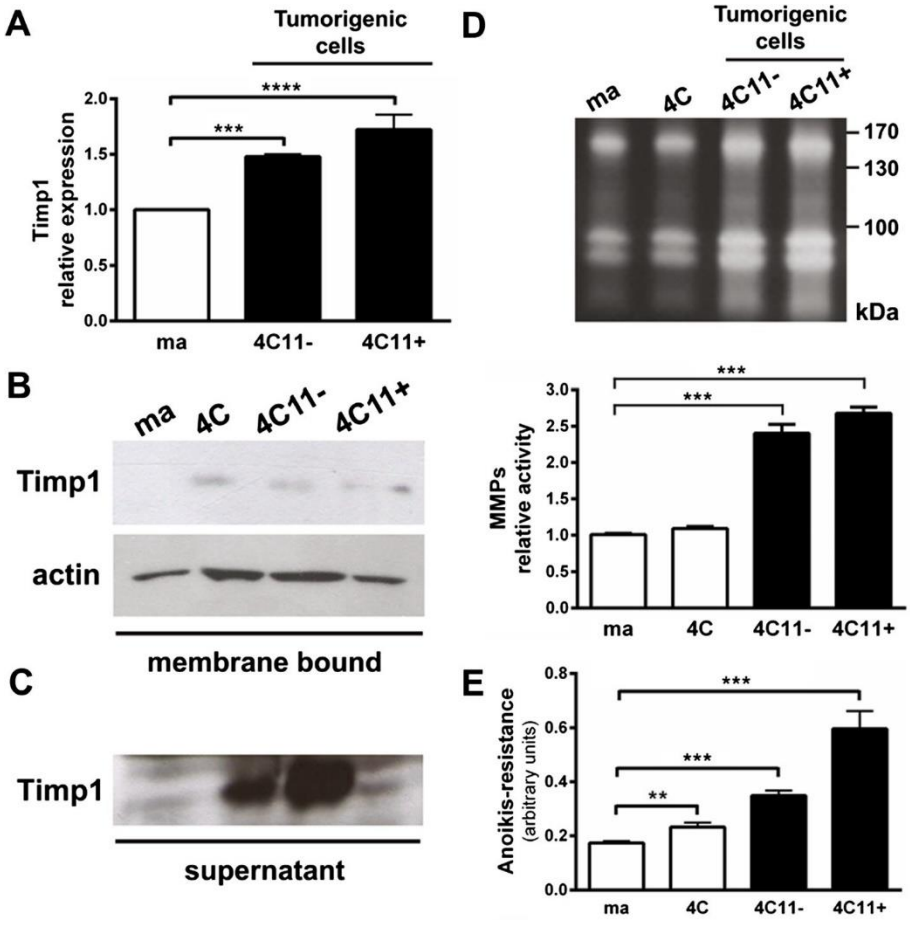

Figure 1 Increased association of Timp1 on the cell surface along melanoma progression. A. mRNA levels of *Timp1* were determined by real time PCR. B. Membrane-enriched extracts were separated on 15% polyacrylamide gel SDS/PAGE and transferred to a PVDF membrane. The membrane was incubated with polyclonal antibody specific to Timp1. The constitutive expression of β-actin was used as endogenous control. C. The supernatants of cell cultures were collected and lyophilized. The presence of soluble Timp1 was identified by Western Blotting. D. Conditioned media (10 μg) from melan-a, 4C, 4C11- and 4C11+ cell lines were evaluated for MMPs activity. E. The same cell lines were maintained in suspension for 96 hours and viable cells were estimated using MTT. Experiments were always performed in quadruplicates. ma: murine non-tumorigenic melanocyte lineage; 4C: pre-malignant melanocytes; 4C11-: non-metastatic melanoma cells; 4C11+: metastatic melanoma cells. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.