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Cardosins improve neuronal regeneration after cell disruption: a comparative expression study

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Abstract The establishment of primary cell cultures is invaluable for studying cell and molecular biological questions. Although primary cell cultures more closely resemble and function like in the native environment, during the culture establishment the cells undergo several changes including the damage sustained during their removal from original tissue. The resultant cells have to rebalance the expression of their processing molecules to ascertain matrix signalling that ensure cell adaptation and consequent proliferation. Hence, we used cardosin, a novel plant enzyme for tissue disaggregation, for isolating and culturing neuronal cells from embryonic rats. The present investigation reports the molecular events, mainly related with matrix metalloproteinases (MMPs)/tissue inhibitor of metalloproteinase (TIMPs) expression, which could substantiate the superior neurite outgrowth and dendritic extension previously described. It was observed that 24 h after primary culture establishment, MMP-2 and MMP-9 messenger RNA (mRNA) are significantly upregulated, while the expression of TIMP-1 and TIMP-2 is unaltered. Regarding the role of laminin in neuronal pathfinding, it was found that the use of anti-laminin antibody and arginine–glycine–aspartate (RGD) peptide exerted inhibitory effects on neurite outgrowth after mechanical lesion where the expression of MMP-9 and TIMP-1 is upregulated under non-permissive conditions in response to mechanical injury.

Keywords Cardosins · Laminin · MMPs · Neurite outgrowth · Neuronal cultures

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Introduction

After neuronal disaggregation, axonal regeneration requires extensive growth cone motility to generate specific features of tissue architecture. Extracellular environmental signals, also known as guidance cues, can be fixed in place or diffusible; they can attract or repel axons (Monard 1988; Fambrough et al. 1996).

Recent data indicate that extracellular matrix (ECM) molecules may activate intracellular signalling cascades and thereby modulate cellular attachment, motility and neuronal outgrowth (Gruenbaum and Carew 1999). ECM signalling is initiated by the interaction of distinct ECM sequences with specific cellular receptors. The best described group of ECM receptors is the integrin family that can interact with a number of ECM components (Schwartz 2001; Schwartz et al. 1995; Campus 2005; Stupack 2005). The interaction can involve different peptide sequences within the ECM molecules, among them the RGD sequence (Arg–Gly–Asp) has been reported to be present in laminin, fibronectin and collagen IV (Yamada 1991; Ruoslahti 1996; Tucker et al. 2005).

It has long been conjectured that proteolytic activity on neuronal growth cones controls their migratory activity (Aguayo et al. 1981; Dzwonek et al. 2004). It is believed that the main function of proteases is to create penetrable paths for axon extension and to modulate the activities of receptors and ligands through proteolytic processing (Dzwonek et al. 2004). Matrix metalloproteinases (MMP) comprise one of the most important families of proteinases that participate in both the processing of bioactive molecules and in the degradative aspects of several diseases (Leppert et al. 2001). The specific inhibition of MMPs by the tissue inhibitor of metalloproteinase (TIMP) family can regulate the extracellular activity of MMPs (Brew et al. 2000). Activation of MMP zymogens is another critical aspect of the regulation of connective tissue matrix composition, structure and function.

Enzymatic tissue disaggregation represents a conditioned step of cell isolation as ECM digestion is required. Hence, it is our purpose to evaluate cellular skills for ECM reexpression after embryonic rat brain dissociation. In a previous work we showed that cardosins, plant aspartic proteinases of *Cynara cardunculus* L. with collagenolytic activity, can be used on the establishment of primary neuronal cultures. These neurons exhibit normal morphology and function when compared to cultures prepared by the widely used trypsin dissociation method. This new protocol, when compared to the classic protocol, shows an improvement on neuronal recovery after cell plating, suggesting that cardosin dissociation of brain tissue is less aggressive to neurons than trypsin (Duarte et al. 2007). As a matter of fact, we showed that cells isolated with cardosins appear to recover faster than those isolated with trypsin, as assessed by the number and length of neurites. The number of neurites extending longer than 25 mm was 68.0±5.0 in cells that had been isolated using cardosins, which is significantly larger than in trypsin isolated cells (39.5±4.0, $P \leq 0.01$). Their maximal length was 35.0± 4.0 mm in trypsin-generated cultures, and 50±6.0 mm ($P \leq 0.01$) in cultures obtained with cardosins. No differences were perceptible after 7 days in culture (Duarte et al. 2007). These aspartic proteinases exhibit a narrow specificity cleaving peptide bonds between hydrophobic amino acids (Sarmento et al. 2004). During the isolation procedure, the collagenolytic activity of cardosins (Duarte et al. 2005) can promote brain disaggregation while avoiding neuronal damage.

In the present work, we report the expression pattern and enzymatic activity of MMP-9 and MMP-2 in primary cultures and address the changes that these enzymes undergo during cell development in culture. Furthermore, we correlated their expression with TIMPs expression. Since laminin constitutes an important substrate for MMPs and mediates several important cell signalling pathways, we further use function-blocking antibodies to address the role of laminin on neurite outgrowth. Trying to elucidate further the mechanisms of the interaction between cortical neurons and ECM, we additionally used RGD peptides, which interfere with adhesion and outgrowth. This work aims to obtain information about matrix reexpression after cell isolation procedure using cardosins that could explain the fast neuronal recovery observed previously.

Material and methods

Reagents Neurobasal medium, B27 supplement and trypsin (USP grade) were purchased from GIBCO. Mouse anti-MAP-2, glutamine, DNase (DN-25), hydrogen peroxide and trypan blue were purchased from Sigma Chemical Co. Goat anti-mouse IgG antibody conjugated to Alexa Fluor® 594 and goat anti-rabbit IgG antibody conjugated to Alexa Fluor® 488 were purchased from Molecular Probes. [3H] GABA was purchased from Amersham, and Universol scintillation cocktail from ICN. Rabbit polyclonal anti-laminin, L9393 was purchased from Sigma and mouse monoclonal anti-MMP-9, Ab-5 (IIA5) was obtained from NeoMarkers. Rabbit polyclonal anti-

MMP-2 (H-76), sc-10736 and rabbit polyclonal anti-β-actin (H-300) sc-10736 were purchased from Santa Cruz Biotechnology. All other reagents were from Sigma Chemical Co. or from Merck KGaA.

Cardosins purification Cardosins were purified according to Sarmento et al. (2004), by a method that allows the purification of high amounts of protein. Briefly, stigmas from fresh flowers were homogenised in a mortar and pestle in sodium citrate 100 mM, pH 3.5, and centrifuged. The supernatant was applied to a Hiload Superdex 75 semi-prep (Amersham Pharmacia Biotech) equilibrated and eluted with 25 mM Tris–HCl, pH 7.6, at a flow rate of 3 ml/min. Purity of cardosins was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) after staining with Comassie Brilliant Blue. Cardosins solutions were concentrated by lyophilisation. Dried protein was stored at −80 °C.

Cell isolation and culture Primary cultures of brain cortical cells were prepared from Wistar rat embryos (E15–E16) as described by Duarte et al. (2007). Animals were handled following the approved guidelines of national ethical requirements for animal research and in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. Briefly, pregnant females were sacrificed by cervical dislocation, the uterus removed under sterile conditions and the embryos dissected on a 100-mm Petri dish with cold Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (CMF-HBSS), pH 7.4. The cortices were carefully removed and placed in CMF-HBSS containing 0.3% of BSA in a 35-mm Petri dish for subsequent removal of the meninges. After slightly minced, the cortices were digested with 0.1% of trypsin or cardosins (concentrations indicated in the figure legends) in CMF-HBSS containing 0.008% Dnase I, for 10 min at 37°C. The digestion was stopped by adding 10% fetal calf serum (FCS) and washing. The digested tissue was mechanically dissociated by gentle forcing through a 5-ml glass pipette. After centrifugation (140×*g*, 5 min), the cells were resuspended in Neurobasal Medium supplemented with 2% B27, 0.5 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell viability was assessed by trypan blue exclusion and counting on a hemocytometer. The cells were plated on poly-D-lysine (0.1 mg/ml) coated multi-well plates, at a density of 0.5×10^6 cells/cm^2, or 10-mm glass coverslips at a density of 0.25×10^6 cells/cm^2. The cells were maintained at 37°C in a humidified 5% CO2/95% air atmosphere.

Immunocytochemistry Cell identity and morphology were evaluated after the immunocytochemical labelling of the cortical neurons with an anti-MAP-2 (micotubule-associated protein 2) antibody, and for the astrocytes with an anti-GFAP (glial fibrillary acidic protein) antibody. After removal of the culture medium and washing with phosphate-buffered saline (PBS), the cells were fixed with 4% paraformaldehyde in PBS for 10 min, followed by permeabilisation with 0.2% Triton ×-100 in PBS for 10 min. After blocking for 90 min with 0.2% gelatine in PBS, the cells were incubated with the anti-MAP-2 (1:500) and anti-GFAP (1:200) antibodies for 90 min. After extensive washes in PBS, the cells were incubated for 60 min with the secondary antibodies: anti-mouse IgG conjugated to Alexa Fluor 594 (2 mg/ml) and anti-rabbit IgG conjugated to Alexa Fluor 498 (1 mg/ml). To assess the specificity of the immunostaining, the primary antibodies were omitted in some coverslips. Finally, the coverslips were washed thoroughly and mounted on glass slides in fluorescent mounting medium (DAKO). The preparations were observed in a Carl Zeiss fluorescence microscope, and images were acquired with the AxioVision software (Carl Zeiss Imaging Systems). The number of neurites and their maximal length were evaluated in four randomised areas, in three independent preparations, with the AxioVision software, and statistically evaluated with Student's *t* test.

RNA preparation and RT-PCR Total RNA was prepared from neuronal cultures using the TRIzol® method according to the manufacturer's protocol (Life Technologies). An aliquot (1 mg of RNA) was used for reverse transcriptase polymerase chain reaction (RT-PCR) using the First Strand cDNA Synthesis Kit for RT-PCR (Roche Molecular Biochemicals). The reverse transcription (RT) reaction conditions were 25°C for 10 min, 42°C for 60 min and 99°C for 5 min.

Specific primer pairs were designed based on sequences deposited in databases having in mind co-amplification with β-actin (used as an internal

standard). The following oligonucleotide primer sequences were used: MMP-2 (sense, 5'-gattgacgc tgtgtatgagg-3'; antisense, 5'-agtctgcgatgagcttagg-3'), MMP-9 (sense, 5'-tgtaccgctatggttacac-3'; antisense, 5'-tccgcgacaccaaactgg-3'); TIMP-1 (sense, 5'-ccacct tataccagcgttatg-3'; antisense, 5'-gaacagggaaacactgtg ca-3'); TIMP-2 (sense, 5'-gcagataaagatgttcaaagg-3'; antisense, 5'-cagtccatccagaggcac-3') and β-actin (sense, 5'-gactacctcatgaagatcct-3'; antisense, 5'-atcttg atcttcatggtgctg-3'). PCR program for MMP-2, TIMP-1 and TIMP-2 was as follows: 94°C, 30 sec; 55°C, 30 sec; 72°C, 1 min in a total of 30 cycles, being the first denaturation step of 2 min and the last extension step of 5 min. For MMP-9, the program was similar except for annealing temperature of 57°C and the number of cycles of 34. The annealing temperature and cycle numbers were chosen such that both the target gene and the β-actin PCR products would be in the linear phase of amplification and of similar intensity. PCR products were electrophoresed in 2% agarose gel in TAE buffer and visualized with ethidium bromide luminescence. Results were quantified using NIH image software (http://rsb.info.nih.gov/nih-image/download.html) by measuring DNA band intensity from digital images taken on GelDoc (Bio-Rad) with Quantity One program. The nucleotide composition of the PCR amplicons was determined by DNA sequence. NCBI databases were used to verify primer specificity and to check the fidelity of amplification by comparing the obtained sequences to the deposited sequences of genes under study.

Western blot analysis Western blots were performed on cell extracts. Cells were collected from three different cultures in each condition and analyzed in triplicates. Each culture dish was rinsed three times with Hank's balanced salt solution (HBSS) at 4°C. The cells were collected by scraping into an extraction buffer containing 25 mM Tris–HCl, 2.5 mM EDTA, 2.5 mM EGTA, 1% Triton ×-100, supplemented with 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 55 μM leupeptin, pH 7.4 at 4°C. Protein concentration was determined using a BCA Protein assay kit (Pierce) and 35 μg of extract were boiled for 5 min after adding 2× concentrated sample buffer [100 mM Tris-Bicine, 2% β-mercaptoethanol, 2% sodium dodecyl sulfate (SDS)]. Equal amounts of protein were separate by electrophoresis on a 7.5% SDS-polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose membranes. These were then blocked for 1 h at room temperature in 5% skim milk (*w*/*v*) in Tris-buffered saline (TBS; 137 mM NaCl, 20 mM Tris–HCl, pH 7.6) containing 0.1% Tween 20 (TBS-T) before immunoblotting. Blocked membranes were incubated in TBS-T overnight at 4°C with primary antibodies (rabbit polyclonal anti-laminin 1/500, rabbit polyclonal anti-MMP-2 [H-76] 1/200, mouse monoclonal anti-MMP-9 1/500 and rabbit polyclonal anti-β-actin [H-300] 1/500). Rabbit polyclonal antibody against *β*-actin was used as a loading control for Western blotting. After three washes with TBS-T, the membranes were incubated for 2 h at ambient temperature with the HRP-labelled second antibody, diluted 1:1000 in TBS-T. The membranes were then washed three times with TBS-T and detection was carried out with enhanced chemiluminescent (ECL) reagents (Amersham Pharmacia Biotech) following the manufacturer's instructions.

MMPs Activities Conditioned media of cell cultures were collected at 24 h, 4 and 7 days after culture establishment and analyzed by gelatin zymography (Liotta and Stetler-Stevenson 1990). Briefly, media samples containing 100 μg of protein were mixed with 2× SDS sample buffer (0.25 M Tris [pH 6.8], 5% [*w*/*v*] SDS, 20% glycerol) and electrophoresed directly without boiling, under nonreducing conditions, on 10% polyacrylamide gels containing 0.1% gelatin. The gels were renatured at room temperature for 30 min in a 2.5% *v*/*v* Triton ×-100 solution, before incubating at room temperature for 30 min in developing buffer (50 mM Tris [pH 7.6], 0.02 M NaCl, 5 mM $CaCl_2$). Gels were incubated in fresh developing buffer overnight at 37°C. The gels were stained (1% Coomassie Blue R-250, Sigma), distained in 25% methanol, 10% acetic acid and MMPs activity demonstrated by the presence of clear bands upon a blue background.

Quantification of gelatinolytic activities, active MMP-2 and transcripts expression was performed on at least four independent experiments. mRNA expression and gelatinolytic activities on zymograms were expressed as a percentage of the controls ±SEM. Student's t tests were used for statistical analysis. Rabbit polyclonal anti-MMP-2 (H-76, Santa Cruz Biotechnology) 1/200 and mouse monoclonal anti-MMP-9 (Ab5 NeoMarkers) 1/500, were used for

gelatinases identification in parallel western blot analysis.

Lesion and culture treatments Embryonic rat neurons were cultured for 7 days to allow neurons to elaborate neurites. Mechanical lesions were carried out by the stylet transection method. Briefly, we used a plastic stylet to scrape adherent cells from a culture dish thereby tearing processes and soma while leaving a significant proportion of cells intact (Morrison et al. 1998). All culture treatments were performed 24 h post-lesion, by adding a pan-laminin antibody (1:200) or RGD peptide (50 μM) to the culture medium. The effects of RGD peptide and anti-laminin antibody on neurite outgrowth were determined both by analysing the number of neurons with neurites close to the scratched vicinity and by measuring the neurite length in four randomised areas, in two independent assays, with the AxioVision software. Neurites of growing neurons were visualized after immunolabelling as described under immunocytochemistry.

Data analysis Expression and activity data are expressed as means ± SEM. Statistical significance was determined by using Student's *t* tests (*$p \leq 0.05$, **$p \leq 0.001$ and ***$p \leq 0.001$, as indicated in the figure legends). Neurite length and number were analysed for differences with one-way analysis of variance (ANOVA), followed by a post-hoc Tukey HSD test, using a significance level of 0.05 (differences between treatments are represented by different letters in the graph).

Results

Expression and activity of ECM proteins in neuronal cultures

In a previous work we reported that cardosins can be used to dissociate embryonic cortex to prepare neuronal cultures exhibiting normal morphology and function compared to cultures prepared by the widely used trypsin dissociation (Duarte et al. 2007). Actually, as shown in Fig. 1, neurons isolated with cardosins and cultured for 24 h after plating consistently exhibited longer neurites (50±6.0 mm, $P \leq 0.01$), compared to the cultures of cells dissociated with trypsin (35.0±4.0 mm, $P \leq 0.01$), but the differences were no longer apparent in cells cultured for longer periods of time (Duarte et al. 2007).

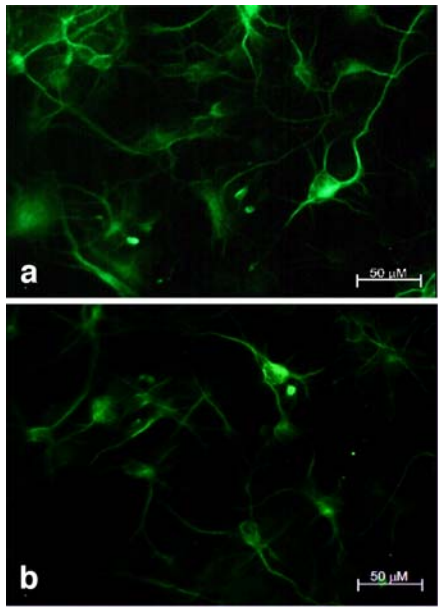

Fig. 1 Morphological characterisation of cell cultures prepared by cardosins (**a**) or trypsin (**b**) dissociation of embryonic brain cortex. The cells were cultured for 1 day before fixation and immunolabelling for MAP-2 (scale bar=50 μM)

Since neuronal growth and development, in particular neuritogenesis, are closely related with proteolytic activity of the ECM, we analysed the activity and expression of MMPs in neuronal cultures prepared from embryonic rat cortex. MMPs are expressed and secreted as inactive precursors that are activated by the removal of an N-terminal propeptide (Okada et al. 1988). The latent and active forms of each enzyme can be differentiated on the basis of their molecular weights using zymography. We observed that the main gelatinolytic activity present in culture media corresponds to the active form of MMP-2. Figure 2 displays a zymography gel showing a higher gelatinolytic activity after 24 h in culture in neurons isolated with cardosins in comparison with the activity of neurons obtained with trypsin. On these gels, we did not detect gelatinolytic activity corresponding to the 92-kDa type IV collagenase, MMP-9.

In addition to the regulation by zymogene activation, most MMPs are regulated at transcriptional level, and their activity is also regulated by specific inhibitors (TIMPs). Hence, we examined the expres-

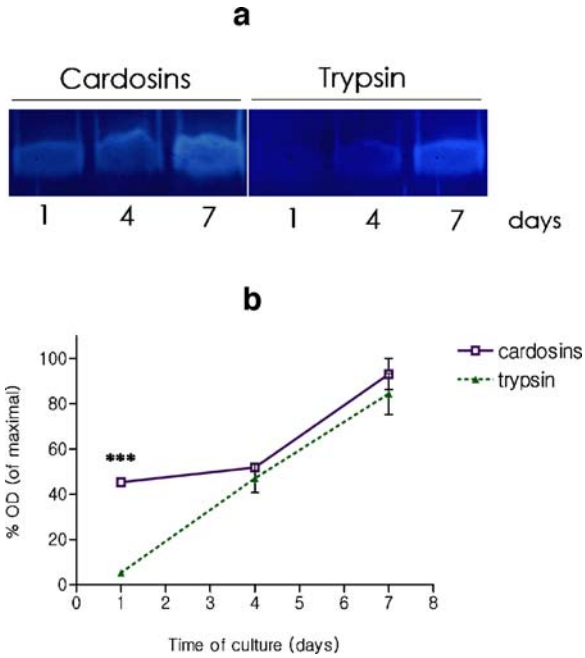

Fig. 2 MMP-2 zymograms of conditioned media from primary neuronal cultures prepared by using cardosins or trypsin. Conditioned media (100 μg) were analysed at 1, 4 and 7 days after culture establishment. **a**) Representative zymograms; **b**) densitometric data are presented as the percentages of maximal activity observed for conditioned media of cultures prepared with cardosins after 7 days in culture. The results (mean ± SD) shown are from five independent tissue samples. ***$P<0.01$, as compared to trypsin

sion of MMP-2 and MMP-9 and of their natural inhibitors, TIMP-2 and TIMP-1, in neuronal cultures using semi-quantitative RT-PCR. Interestingly, multiplex RT-PCR showed significant differences in the expression of MMP-2 and MMP-9 on the first day of culture in the two types of cell preparations (Fig. 3). The neuronal cultures established with cardosins showed significant higher levels of MMP-2 and MMP-9. There are no major differences on the expression after 4 and 7 days on culture for both gelatinases. The same is observed for TIMP-2 expression that is equally expressed during all experiments. In contrast, the increasing pattern of TIMP-1 mRNA expression is lost after 7 days. As presented in Fig. 3b, TIMP-1 expression in neurons isolated with trypsin is markedly high compared to cultures established with cardosins.

To get further insight on the expression of ECM components in neuronal cultures, MMPs and laminin protein levels were measured by western blotting, using β-actin as a loading control (Fig. 4). The results for MMP-2 and MMP-9 are consistent with the data of mRNA levels since the major differences were found on the first day of culture. In fact, the protein expression for both gelatinases is much higher for neuron isolated with cardosins after the first 24 h. Laminin is an important ECM protein that has been found to be involved in cellular activities such as neuronal migration and neurite outgrowth. A high level of laminin constitutes a key permissive element that can be modulated by proteolytic activity (Costa et al. 2002). The immunoblotting analysis detected a 200- to 220-kDa protein band expected for the size of laminin β/γ chains. Generally, neurons isolated with cardosins and cultured for 24 h express higher levels of laminin than neurons isolated with trypsin, although after 4 and 7 days in culture laminin level were not significantly different in the two types of cultures, suggesting that laminin level is an essential element for cell recovery in both isolation procedures.

Anti-laminin effect on permissivity to neuritogenesis

To examine the ability of both culture types to respond to injury, we mechanically lesioned 7-day-old neuronal cultures and we tested the effect of an anti-laminin antibody on neurite outgrowth in the vicinity of the lesion. This treatment was reported by Costa et al. 2002 to block neurite outgrowth and to induce an apparent inhibition of neuronal migration toward the lesion site. This is corroborated by our results (Fig. 5), where anti-laminin antibody has significantly reduced neurite sprouting.

It has been described that both TIMP-1 and TIMP-2 display growth promoting in a wide variety of cell types (Hayakawa et al. 1994; Bertaux et al. 1991; Stetler-Stevenson et al. 1992). Our results of mRNA expression 24 h after lesion show that treatment with anti-laminin antibody led to upregulated TIMP-1 and MMP-9 expression (Fig. 6a), while differences on TIMP-2 and MMP-2 mRNA expression were not detected (data not shown). In addition, no differences in MMP-2 activity were detectable on zymography gels.

Cell responses to injury on both culture types were comparable for mRNA expression of MMPs/TIMPs and for activity, suggesting equal cell morphology and functionality after 7 days in culture. Peptides containing RGD sequence can be used to block cellular adhesion to RGD sequences within substrata.

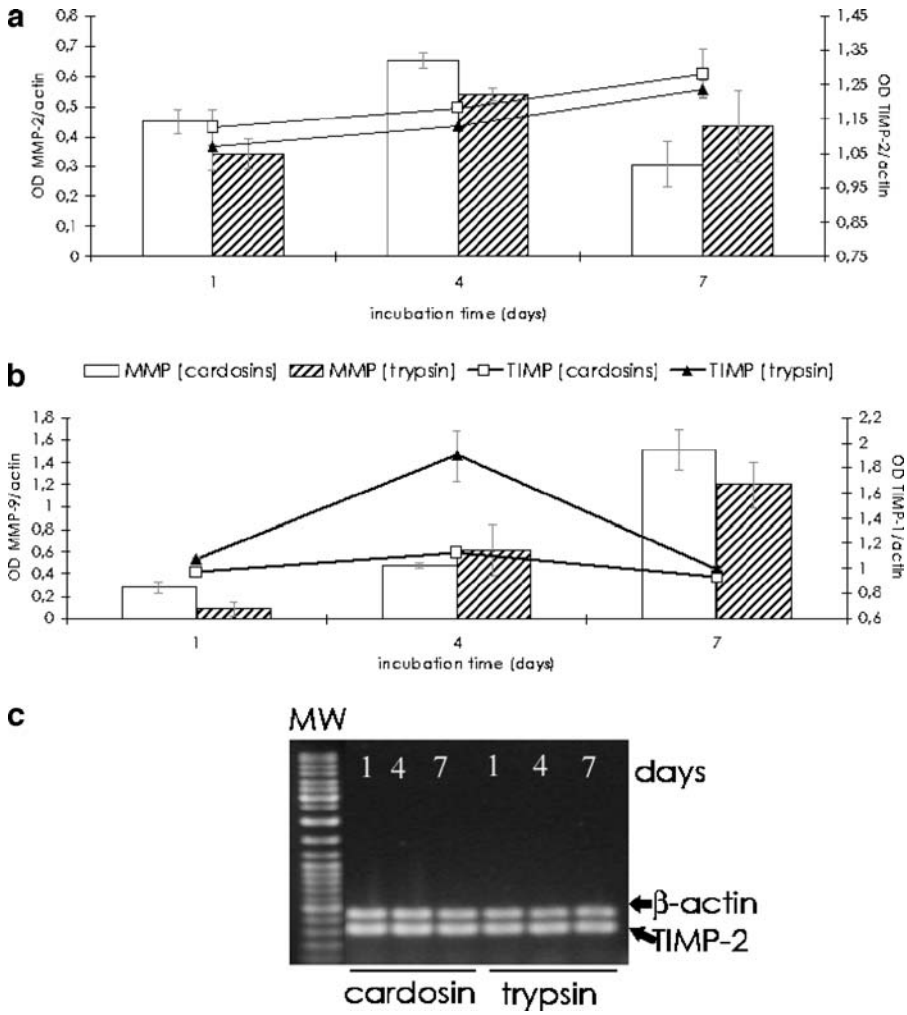

Fig. 3 Expression of MMPs and TIMPs on neuronal cultures prepared with cardosins or trypsin. **a**) mRNA of MMP-2 and TIMP-2; **b**) mRNA of MMP-9 and TIMP-1. The values are the ratios of the mRNA band densities of the target gene/β-actin; **c**) Representative gel electrophoresis of TIMP-2 mRNA performed as described under "Material and methods" section; the multiplex RT-PCR produced a 308-base pair TIMP-2 fragment and a 490-base pair β-actin fragment

Similar to integrins, RGD sequences may be involved not only in adhesion, but also in the promotion of neurite outgrowth (Tashiro et al. 1991). Therefore, parallel assays were performed by mechanically treating injury of neuronal cultures with RGD peptide, instead of anti-laminin antibody. Figure 5 shows that after a mechanical injury, RGD effect on neuronal regrowth is similar to the effect observed for the anti-

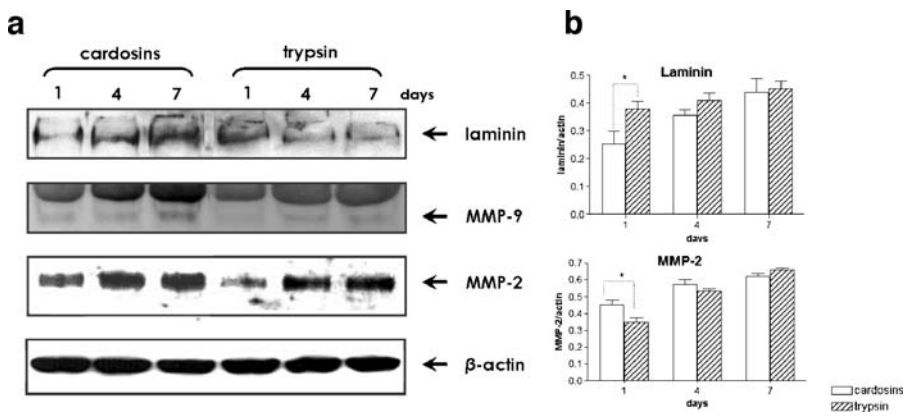

Fig. 4 Levels of laminin, MMP-2 and MMP-9 in extracts of neuronal cultures prepared with cardosins or trypsin. **a**) Representative western blots; **b**) quantification of laminin and MMP-2 using β-actin as a loading control

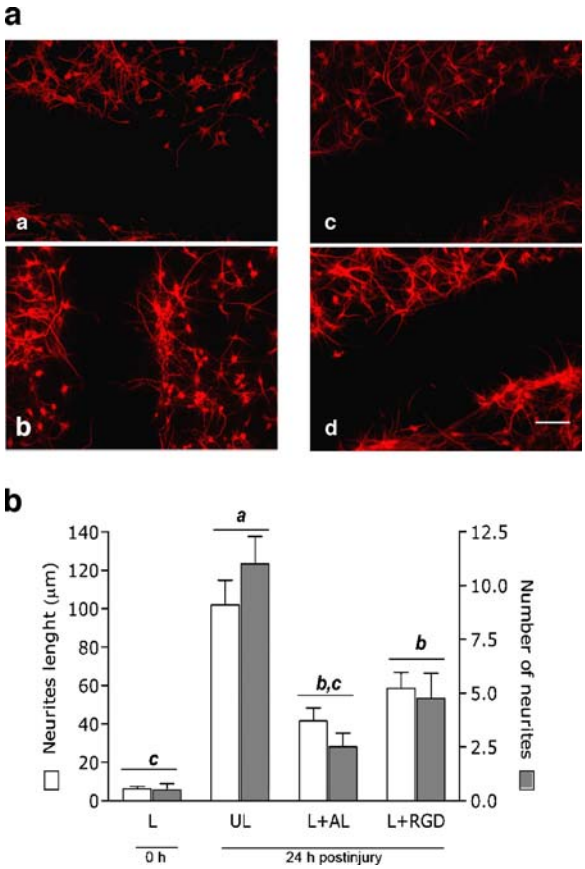

Fig. 5 Neurite outgrowth evaluation after mechanical injury. **a**) Representative immunoscytochemistry analysis: *a*) initial lesion on a 7-day culture; *b*) 24 h postlesion untreated culture. Observe the neurite outgrowth inside the lesion site compared with the control; *c*) 24 h postlesion treated culture with anti-laminin antibody (1/200); *d*) 24 h postlesion treated culture with RGD peptide (500 µM). In *c*) and *d*) the reduction of neurites projecting toward the lesion (scale bar=100 µm) is clear. **b**) Morphometric analysis of neurite length (µm) in lesioned neuronal cultures (left axis), and of the number of neurites, extending longer than 5 µm away from the neuronal border into the lesioned area (right axis), 24 h postinjury. L – lesioned cultures (0 h); UL (24 h) – untreated lesions (control); L + AL (24 h) – treated culture with laminin antibody (1/200); L + RGD – treated culture with RGD peptide (500 µM). Neurite length and number were analysed for differences with one-way analysis of variance (ANOVA), followed by a Tukey HSD multiple comparison test, using a significance level of 0.05 for all analyses. Statistically significant differences between treatments are represented by different letters (*a*, *b*, *c*)

laminin antibody. Similarly, RGD treatment induced MMP-9 and TIMP-1 mRNA expression (Fig. 6a) and no additional differences on MMP-2 were detectable by zymography. In addition, an increase of extracellular laminin level assessed by western blot is clear (Fig. 6b).

Discussion

Extracellular matrix (ECM) plays a key role in mediating neurite outgrowth following brain tissue dissociation for the establishment of primary neuronal cultures. It is well-known that ECM regeneration is achieved by the effect of MMPs activity on matrix or non-matrix proteins performing directly or indirectly on the rebuilding of the new network. Matrix metalloproteinase (MMP) enzymatic activity plays an

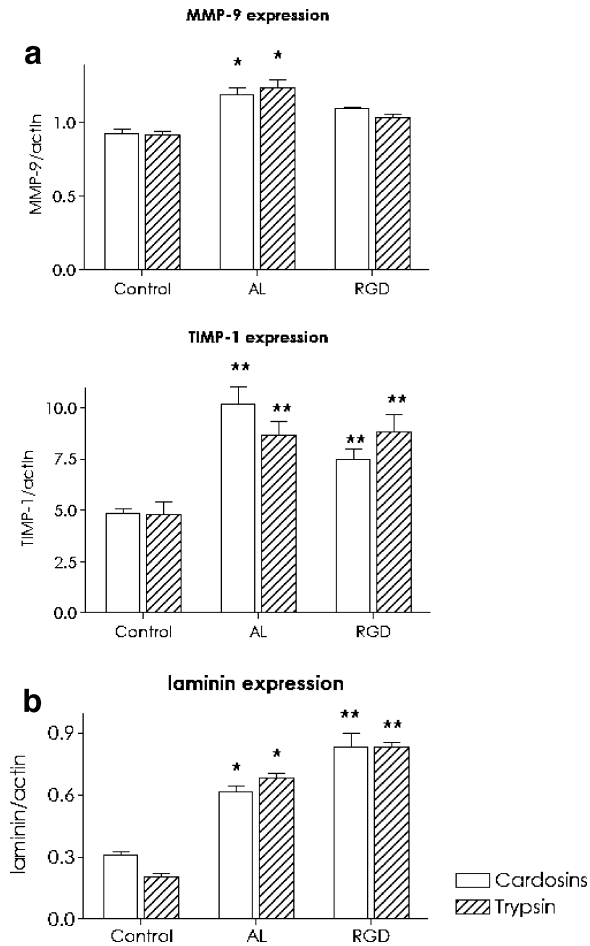

Fig. 6 MMP-9, TIMP-1 and laminin expression in mechanical lesioned neuronal cultures. **a**) Densitometric results from semi-quantitative RT-PCR analysis (mean ± standard deviation). Effect of anti-laminin addition to conditioned medium on mRNA expression of MMP-9 and TIMP-1. **b**) Laminin quantification assessed by western blot analysis. Open bars, cultures established with cardosins; patterned bars, neuronal cultures established with trypsin (n=3). $^{**}P<0.01$, $^{*}P<0.05$. All the described analysis were performed in parallel on both culture types (cardosins *versus* trypsin) showing the same results

important role in brain physiology, possibly consisting of pivotal significance for neuronal plasticity. The focus of our investigation consisted of the study of the expression pattern and enzymatic activity of these molecules in neuronal cultures established with cardosins, to elucidate the molecular events responsible for the observed faster neuronal recovery, compared to trypsin. Previous studies by Reeves and colleagues suggested a potential functional role of MMP-2 in post-lesion axonal sprouting and neuronal plasticity. They show that differentiation and subsequent functional plasticity recovery is correlated to an increased MMP-2 activity (Reeves et al. 2003). Similarly, during brain disaggregation neurons are strained because of disruption of connections to the neighbouring cells triggering a recovery-like cell response (Pérez-Martínez and Jaworski 2005). In the present work we show that after 1 day in culture, MMP-2 expression and activity levels are higher for neurons isolated with cardosins compared to trypsin. These observations substantiate previous remarks about early cell recovery as assessed by morphological evaluation.

Moreover, the primary function of TIMPs is to inhibit MMP activity, consequently TIMP-1 and TIMP-2 expression may be upregulated simply in response to increased MMP expression. TIMP-1 expression is associated with neuronal plasticity (Dzwonek et al. 2004) and neurons isolated with trypsin have a less prompt recovery to the isolation procedure, in contrast to those isolated with cardosins (Duarte et al. 2007). This may be the reason for the huge TIMP-1 mRNA expression observed at the fourth day of culture for neurons obtained with trypsin.

It is generally acknowledged that neuronal response to CNS insults operates by selective enhancing of MMPs activities that promote recovery mechanisms and initiate neuroprotective pathways. In this study, the neuronal response to mechanical injury, correlated with MMP-9 and TIMP-1 upregulation. Although it is not yet clear whether MMP-9 is expressed by the neurons prone to death and/or those attempting to recover from the damage, a potential correlation has been proposed between neuronal survival, synaptogenesis and MMP-9 (Dzwonek et al. 2004) supporting the presented data.

Matrix metalloproteinase (MMPs) are regulated after some types of insult, such as cell isolation or mechanical lesion (Yong et al. 2001). With respect to cell migration, neural cell precursors may require a rebalance of MMPs activities to migrate to the scarred area. New axonal growth and synapse formation need to be established and their extension through the brain matrix may also require a new MMPs/TIMPs ratio. This may be the basis for an increase of MMP-9 and TIMP-1 expression evident in this work. This new balance must contribute directly or indirectly to the increased laminin expression, which assists regrowth.

The effects of the pan-laminin antibody and the RGD peptides on the morphology of cortical neurons were also investigated. The anti-laminin antibody modulated neuronal morphology in a pattern very similar to the effects of RGD sequence: both treatments led to a decrease of neurite outgrowth in the scarred area (Fig. 5). Moreover, as shown in Fig. 6b, RGD treatment promoted laminin overexpression. One speculation arising from these observations is that both types of culture (established with cardosins or with trypsin) respond to stress/injury conditions via laminin expression, a well-known mechanism that favour permissivity. Finally, these data suggest that cortical neurons use RGD-dependent mechanisms for adhesion and outgrowth. These mechanisms provide possible sites of modulation by specific growth factors, as ECM components can act not only as passive substrates for neuronal attachment and outgrowth, but also as active sites for signal transduction.

In conclusion, present results indicate that cells respond to isolation procedure by upregulating both gelatinases, MMP-2 and MMP-9, agreeing with our prior observation of a faster neuronal outgrowth. After mechanical injury, it was also shown an inhibition of neuronal recovery, as assessed by morphological analysis, together with an increase of TIMP-1 and MMP-9 mRNA levels under non-permissive conditions (AL treatment). RGD-containing peptide had significant influence in the upregulation of TIMP-1 and laminin. Taken together, these approaches indicate that the regulation of MMPs levels can help to predict the cellular state, suggesting that MMPs should be viewed, not only as proteolytic enzymes acting on ECM remodelling but, overall, performing as pruning shears, playing sophisticated roles in modulating normal cellular behaviour, cell–cell communication and tumor progression. Moreover, the results emphasise the significance of the enzymatic influence evaluation on isolation procedures, given that different enzymes can produce divergent effects on cell morphology and protein expression by stimulating

or inhibiting cell response. However, after 7 days cardosins and trypsin produce equivalent cell preparations; cardosins certainly improve neuronal regeneration at early stages of culture after cell isolation. This feature might be particularly useful for research purposes requiring fast cell manipulation.

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