CHAPTER 2

THE ACIDIC DOMAIN I OF SPARC/ BM40/ OSTEONECTIN DOWN-REGULATES EXTRACELLULAR TIMP-2 INDEPENDENT OF MMP-2 ACTIVATION

Summary

SPARC (secreted protein acidic and rich in cysteine) is a matricellular protein important for many normal and pathologic processes. Our previous study [36] showed that recombinant human SPARC could induce activation of matrix metalloproteinase-2 (MMP-2) and reduce levels of tissue inhibitor of metalloproteinase-2 (TIMP-2) in the conditioned media of breast cancer cell lines. In the present study, we compared the effects of different preparations of SPARC from various species (human, bovine and mouse) on the BT-549 human breast cancer cell line. All full length SPARC preparations used in this study reduced TIMP-2 levels in the conditioned medium, however this was not accompanied by increased TIMP-2 in cell lysates. By using domain mutants, we determined that the amino terminal domain I was responsible for extracellular TIMP-2 reduction. Not all SPARC preparations could induce activation of MMP-2, however, and those that could were effective to different degrees. When we used anti-TIMP-2 antibody to neutralize soluble TIMP-2, we found that MMP-2 activation was slightly increased with addition of 0.5-5 μg/ml of antibody, but that activation was inhibited with 10 μg/ml. We concluded that reduction of extracellular TIMP-2 by SPARC did not appear to be a consequence of MMP-2 activation, and did not always cause MMP-2 activation. The discordant effects of SPARC on TIMP-2 reduction and MMP-2 activation may be due to altered local concentrations of TIMP-

2. These data emphasise the complex nature of SPARC effects on MMP-2 activation, but strongly implicate the amino terminal domain of SPARC in this process. The actual mechanism of this reduction remains elusive.

Introduction

Tissue remodelling and extracellular matrix (ECM) degradation is important for many normal biological processes and also for tumour progression. Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes involved in these processes [100]. Among the MMPs, MMP-2 (gelatinase A), and also MMP-9 (gelatinase B) are closely linked to tumour progression, and tumour cell invasion and metastasis, since they specifically degrade collagen type IV, a major component of basement membrane [107, 108]. MMP-2 is primarily expressed in mesenchymal cells (mainly fibroblasts) during tissue reorganisation and highly expressed in stromal cells surrounding tumours [111, 112, 183, 184]. Many studies have correlated increased levels of active MMP-2 in cancer with poor outcome [117, 185, 186], and higher levels of MMP-2 have been reported in stromal cells around the invading front of metastasizing tumours [109-112].

In common with most other MMPs, MMP-2 is secreted as a latent proenzyme and requires proteolytic activation. MMP-2 activation primarily occurs at the cell surface through a trimolecular complex of membrane type MMP (MT-MMP), tissue inhibitor of metalloproteinases-2 (TIMP-2) and proMMP-2 [99]. TIMPs are a family of MMP inhibitors with 4 members known as TIMP-1, 2, 3 and 4 [187]. All TIMPs can bind the active site of MMP-2, but MT-MMPs are not inhibited by TIMP-1 [188]. TIMP-2 is somewhat specific for MMP-2, since it can also bind to proMMP-2 through the MMP-2 carboxy terminal hemopexin domain. Since TIMP-2 can act as

both inhibitor and activator of MMP-2, its optimal level is required for normal function. The balance between TIMPs and MMPs precisely regulates the degradation or accumulation of extracellular matrix [189].

Eventhough many breast cancer cell lines expressed both MT1-MMP and TIMP-2, they are unable to activate exogenous MMP-2 constitutively and require additional stimulation. Con A [126], a plant lectin, can increase MT1-MMP mRNA and protein level and also regulate pre-existing MT1-MMP to induce MMP-2 activation *in vitro*. Similarly, fibrillar collagens, which may represent a physiological counterpart to Con A, also regulate the levels and activity of MT1-MMP, and MMP-2 activation in a variety of cancer cell lines, fibroblasts and endothelial cell (reviewed in [136]). Similar effects have been reported for fibronectin but seem specific to HT1080 cells [190]. SPARC

also known as osteonectin or BM-40, is a 43 kDa glycoprotein capable of inducing MMP-2 activation in breast cancer cell line [36]. SPARC is classified as a matricellular protein, since it binds cells to the ECM but is not part of ECM structure itself. It is composed of 3 domains under current nomenclature [4]: an acidic N-terminal domain I, a follistatin-like (FS) domain II and an extracellular calcium binding (EC) domain III at the C-terminal. SPARC has numerous biological effects in various cell types but its principle functions *in vitro* are antiadhesion and antiproliferation [38]. A number of studies showed overexpression of SPARC in many cancers including melanoma [28], hepatocellular carcinoma [29], esophageal carcinoma [30], prostate cancer [31] and breast cancer [32, 33].

Our previous study showed that recombinant human SPARC produced in *Escherichia coli* [191] could induce MMP-2 activation in breast cancer cell lines [36]. This process, although MT1-MMP dependent, did not involve upregulation of MT1-

MMP mRNA or protein, but was associated with a reduction of TIMP-2 in the conditioned medium. Here we have looked more closely at the interplay between levels of soluble TIMP-2 and MMP-2 activation in the BT-549 breast cancer cell line. SPARC preparations from different species were all able to reduce TIMP-2 but variably induced MMP-2 activation. Also, neutralization of TIMP-2 with two different monoclonal antibodies caused biphasic effects on MMP-2 activation. Domain mutants of human recombinant SPARC showed an important role of the acidic amino terminal domain on TIMP-2 reduction, and a lack of concordance between TIMP-2 and MMP-2 activation. The data suggest that MMP-2 activation may occur as a consequence of the reduced TIMP-2 levels.

Experimental procedures

Cell culture and reagents

BT-549 and MDA-MB-231 human breast cancer cells, originally from ATCC (Rockville, MD, USA), were cultivated in DMEM (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal calf serum (DMEM-FCS) (CSL Limited Biosciences, Parkville, Victoria, Australia). Cultures were maintained at 37°C in 5% CO_2. Recombinant TIMP-2 (rTIMP-2) produced in a vaccenia virus expression system [192] was kindly provided by Dr. Rafael Fridman, Wayne State University, Detroit, MI, USA. Monoclonal anti-TIMP-2 antibody (αC-T2) [193] was kindly provided by Dr. Christopher Overall, University of British Columbia, Vancouver, Canada and the clone no. 67-4H11 monoclonal anti-TIMP-2 antibody was purchased from Fuji Chemical Industries, LTD, Toyoma, Japan. Collagen type I (Vitrogen100) was from Cohesion, CA, USA.

SPARC and mutants

The production of recombinant human SPARC [72], the deletion mutants delta I and delta I-II [4], and recombinant mouse SPARC [194] in mammalian cells have been described, as has extraction of mouse SPARC from Engelbreth-Holm-Swarm (EHS) tumour [2]. In addition, we tested a reduced and alkylated form of full length SPARC which disrupts domain II and III (RA), kindly provided by Prof. Rupert Timpl, Max-Plank-Institut Für Biochmie, Germany. Purified human platelet osteonectin and bovine bone osteonectin were purchased from Haematologic Technologies Inc, VT, USA. Mouse SPARC purified from mouse parietal yolk sac (PYS-2) cells was from Sigma, MO, USA. Human peptide 1.1 (H-Ala-Pro-Gln-Gln-Glu-Ala-Leu-Pro-Asp-Glu-Thr-Glu-Val-Val-Glu-Glu-Thr-Val-Ala-Glu-Val-Thr-Glu-Val-NH2) was synthesized by AUSpep, Parkville, Australia, to approximately 91% purity. It was confirmed by amino acid composition analysis (Beckman 6300 Amino Acid Analyser, Beckman Coulter, Inc., CA, USA) in our institute.

Culture conditions

For all experiments, cells were plated overnight in DMEM-FCS. The next day, wells were washed 3 times with DMEM and changed to DMEM supplemented with 0.1% BSA (DMEM-BSA) with 25% serum free media from 72 hours cultured of MMP-2 transfected MCF-7 cells as a source of exogenous MMP-2. At that time, SPARC, its peptide, or various mutants were added to the cells with or without rTIMP-2 or collagen type I. After 72-96 hours, conditioned media were collected for zymography and/or Western analysis.

For anti-TIMP-2 antibody treatment, different concentrations of antibody were added to the cells in DMEM-BSA/MMP-2 for 72 hours. Before conditioned medium

collection, the antibody was pulled down with washed protein A Sepharose slurry (15 µl/100 µl of media: Pharmacia Biotech, Uppsala, Sweden). The samples were incubated with the beads under rotation at 4°C for 1 hour, and the beads removed by centrifugation (2000 RPM, 2 minutes). The supernatant was collected and subjected to zymogram and TIMP-2 Western analysis.

For protein lysate and RNA collection, wells were washed with PBS after incubations with SPARC etc. in DMEM-BSA/MMP-2. Total protein lysates were obtained in RIPA buffer (50mM Tris-HCl, 150mM NaCl, 1mM EDTA, 1% NP-40, 0.25% Na-deoxycholate) containing proteinase inhibitors; 10 µg/µl Aprotinin, 10 µg/µl Leupeptin, 1mM PMSF, 1mM NaF, 1mM NaP and 1mM Na_3VO_4. Protein concentrations were measured using BCA protein assay reagent as per manufacturer's guidelines (Pierce, IL, USA). For RNA collection, total RNA was extracted using RNAzol BTM (TEL-TEST, Inc., TX, USA), concentration determined by UV-visible spectrophotometry (Ultrospec3000, Pharmacia Biotech, Australia), and integrity confirmed by ethidium bromide staining of 18 and 28S bands after electrophoresis in 1% agarose gel.

Gelatin Zymography

Gelatin zymography was used to monitor the degree of MMP-2 activation in the conditioned media as described by Ruangpanit *et al.* [136]. Areas of MMP-2 activity were seen as clear bands against a blue background of stained gelatin and identified on the basis of co-migration with known standards.

RT-PCR for MT1-MMP & TIMP-2

The RNA was first DNase-treated in a 20 µl reaction containing 10 µg RNA, 2 µl PCR buffer (Roche, Germany), 10 U DNase (Roche), 40 U RNase inhibitor (Promega, Madison, WI, USA) and diethylpyrocarbonate (DEPC)-treated water. The mixture was incubated at 37°C for 30 minutes and then heated to 100°C for 10 minutes. First-strand cDNA was synthesized by RT. In a typical 25 µl reaction, 2 µg of DNase-treated RNA, 0.5 µg of oligo d(T) 12-18 (Gibco/BRL, Gaithersburg, MD, USA) and DEPC-treated water were heated to 70°C for 10 minutes then chilled on ice. First strand buffer (5X, 5 µl; Gibco), 2.5 µl of 0.1 M DTT (Gibco), 2.5 µl of 5 mM dNTP (New England Biolabs, Inc, MA, USA), 40 U of RNase inhibitor (Promega) and 200 U superscript reverse transcriptase (Gibco) were added and the mixture incubated at 42°C for 1.5 hours.

PCR amplification was carried out in a GeneAmp 5700 Sequence Detection System (TaqMan; PE Applied Biosystem, Scoresby, Australia). The primers were synthesized by GeneWorks (Adelaide, Australia) and the probes were synthesized by PE Applied Biosystems. The sequence of primers and probes are as follows: for MT1-MMP: forward (MMP14F5) AGTAACAGGCAAAGCTGATGCA; reverse (MMP14R5) CCCCAAACTTGTCTGGAACAC; probe ACACCATGAAGGCCATGAGGCGC: for TIMP-2:forward (Timp2F3) CCTGAACCACAGGTACCAGATG; reverse (Timp2R3) AGGAGATGTAGCACGGGATCA; probe CTGCGAGTGCAAGATCACGCGC.

The PCR reaction was performed in 25 µl of TaqMan universal PCR master mix (PE Applied Biosystems) containing 25 pmole of each primer, 200 nM of the TaqMan probe and 0.8 ng of sample DNA. The PCR conditions were initially 50°C for 2 minutes followed by 95°C for 10 minutes, followed by 50 cycles of 95°C for 15

seconds and anneal/extension at 60°C for 1 minute. The ribosomal protein L-32 was used as an internal control.

Western analysis

Conditioned media or protein lysates were separated under reducing conditions by 10% SDS-PAGE. Loading was standardized by amount of total protein in cell lysate. The proteins were then transferred to PVDF membrane (Immobilon, Millipore Corp., Bedford, MA, USA) for immunoblotting. Transfer was monitored by reversible staining with Ponceau Red (Sigma). The blots were blocked for 2 hours with blocking solution (5% skim milk, 0.05% Tween 20 in PBS pH 7.5) and then incubated with primary antibody in blocking solution overnight at 4°C. The membrane was then washed 3 times for 10 minutes with blocking solution and incubated for 1.5 hours at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody as detailed below. Signals were developed with an enhanced chemiluminescence (ECL) kit according to the manufacturer's instructions (Pierce).

For TIMP-2 detection, anti-hTIMP-2 1.25 μg/ml (clone no.67-4H11: Fuji Chemical Industries, LTD) was used as primary antibody and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Pierce, IL, USA) dilution 1:20,000 was used as the secondary antibody. For SPARC detection, the 996+ rabbit polyclonal antiserum against human SPARC [72] was used as a primary antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Dako, Denmark) dilution 1: 10,000 was used as the secondary antibody. In some cases (where indicated) we also used the monoclonal mouse anti-SPARC (Anti-osteonectin,

Haematologic Technologies Inc.) followed by HRP-conjugated goat anti-mouse IgG antibody.

Results

Characterization of BT-549 and MDA-MB-231 cells

It was previously found that both BT-549 and MDA-MB-231 human breast cancer cell lines responded to SPARC with induction of MMP-2 activation and reduction of TIMP-2 in conditioned medium [36]. BT-549 cells showed better responses to SPARC than the MDA-MB-231 cells. Here, we first compared the levels of endogenous TIMP-2 secreted by these 2 lines using Western analysis of conditioned media. As shown in figure 2.1A, BT-549 cells secreted a higher amount of endogenous TIMP-2 than MDA-MB-231 cells. BT-549 cells also showed better activation of MMP-2 after the 3-day culture without additional treatment, as seen in zymography as three bands; mainly proform (72 kDa), some intermediate (64 kDa) and some fully active form (62 kDa) (figure 2.1B). In contrast, the MDA-MB-231 cells showed mostly pro-MMP-2, and a very faint band of intermediate form.

When exogenous rTIMP-2 (10, 20 and 50 ng/μl) was added to the cultures, constitutive (unstimulated) MMP-2 activation by BT-549 cells was immediately inhibited after addition of the lowest concentration (10 ng/μl) of rTIMP-2 and at all higher concentrations of rTIMP-2. MDA-MB-231 cells did not show constitutive MMP-2 activation, and this was not affected by the added TIMP-2. Both BT-549 and the MDA-MB-231 are known to respond to collagen with induction of MMP-2 activation [135]. When treated with both collagen and increasing concentrations of exogenous rTIMP-2, again activation of MMP-2 by BT-549 cells was immediately inhibited by the lowest concentration of exogenous rTIMP-2, and by all higher

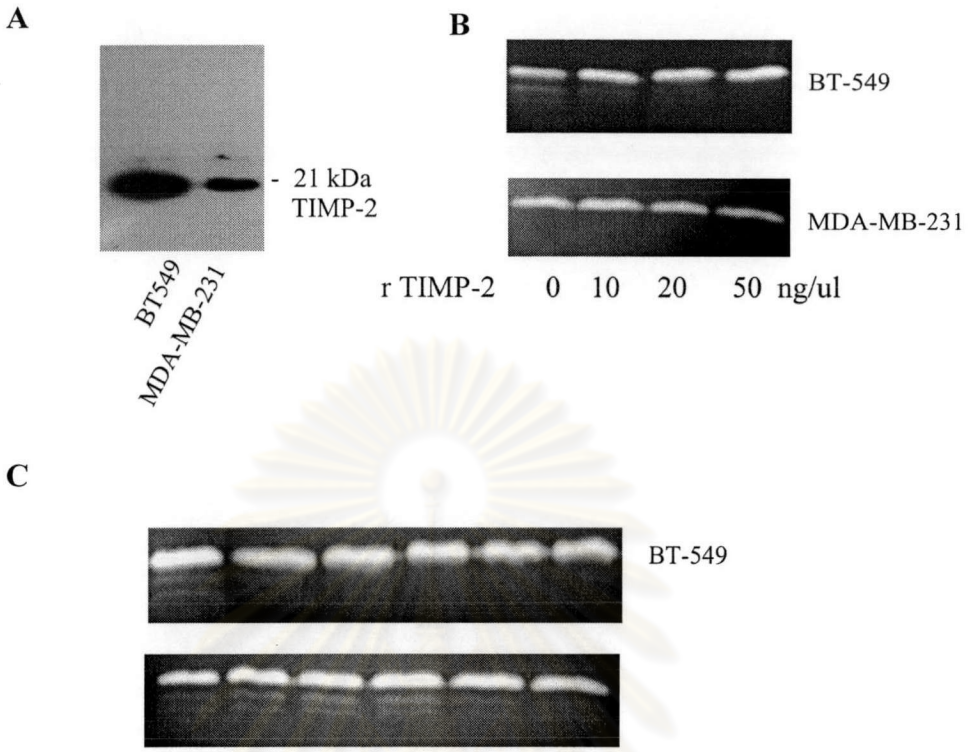

Figure 2.1: Comparison of TIMP-2 levels and MMP-2 activation ability between BT-549 and MDA-MB-231.

A. Cells (8,000) were plated in a 96-well plate, let attach overnight and changed to DMEM-BSA (100 µl/well). After 96 hours, conditioned media were collected and subjected to TIMP-2 Western analysis.

B. and C. Cells (50,000) were plated overnight in a 24-well plate and changed to DMEM-BSA (300 µl/well). Different concentrations of rTIMP-2 were added to the culture either alone (B) or in combination with 50 µg/ml of collagen type I (C). After 72 hours, conditioned media were collected and subjected to zymography.

concentrations. In contrast, MDA-MB-231 cells, which have a lower level of endogenous TIMP-2, were able to tolerate concentrations of added rTIMP-2 up to 75 ng/μl before MMP-2 activation was inhibited (figure 2.1C).

Many studies have shown that optimal levels of TIMP-2 are important for MMP-2 activation and that too much or too little TIMP-2 could inhibit the activation process [123, 195]. Our results showed that BT-549 cells already have high levels of endogenous TIMP-2 compared to the MDA-MB-231 cells. For this reason, we chose BT-549 for studying further the effect of SPARC, which can reduce TIMP-2 levels and potentially lead to MMP-2 activation.

Effects of full length SPARC on MMP-2 activation and soluble TIMP-2 reduction

In this study, we compared the effects of different preparations of SPARC. For full length SPARC, we tested recombinant human SPARC (rhSPARC), SPARC purified from human platelets (hu.plSPARC) or from bovine bone (bovSPARC), SPARC purified from mouse parietal yolk sac (PYS-2) cells (pmSPARC), recombinant mouse SPARC (rmSPARC) and mouse SPARC purified from the EHS tumour (LN81). Western analysis using the 996+ rabbit polyclonal antiserum against human SPARC after SDS-PAGE under reducing conditions showed a discrete 43 kDa band for hu.plSPARC, bovSPARC and rhSPARC (figure 2.2). This antibody was also able to recognize the deletion mutants Del I (~30 kDa) and Del I-II (~14 kDa), and the RA preparation, which had a slower electrophoretic mobility (~50 kDa). With the monoclonal mouse anti-human SPARC, we were also able to detect full length human and bovine SPARC preparations (mouse SPARC not tested), but not deletion mutants Del I and Del I-II, indicating that this antibody recognizes an epitope in the N-terminal domain I of the SPARC molecule (data not shown).

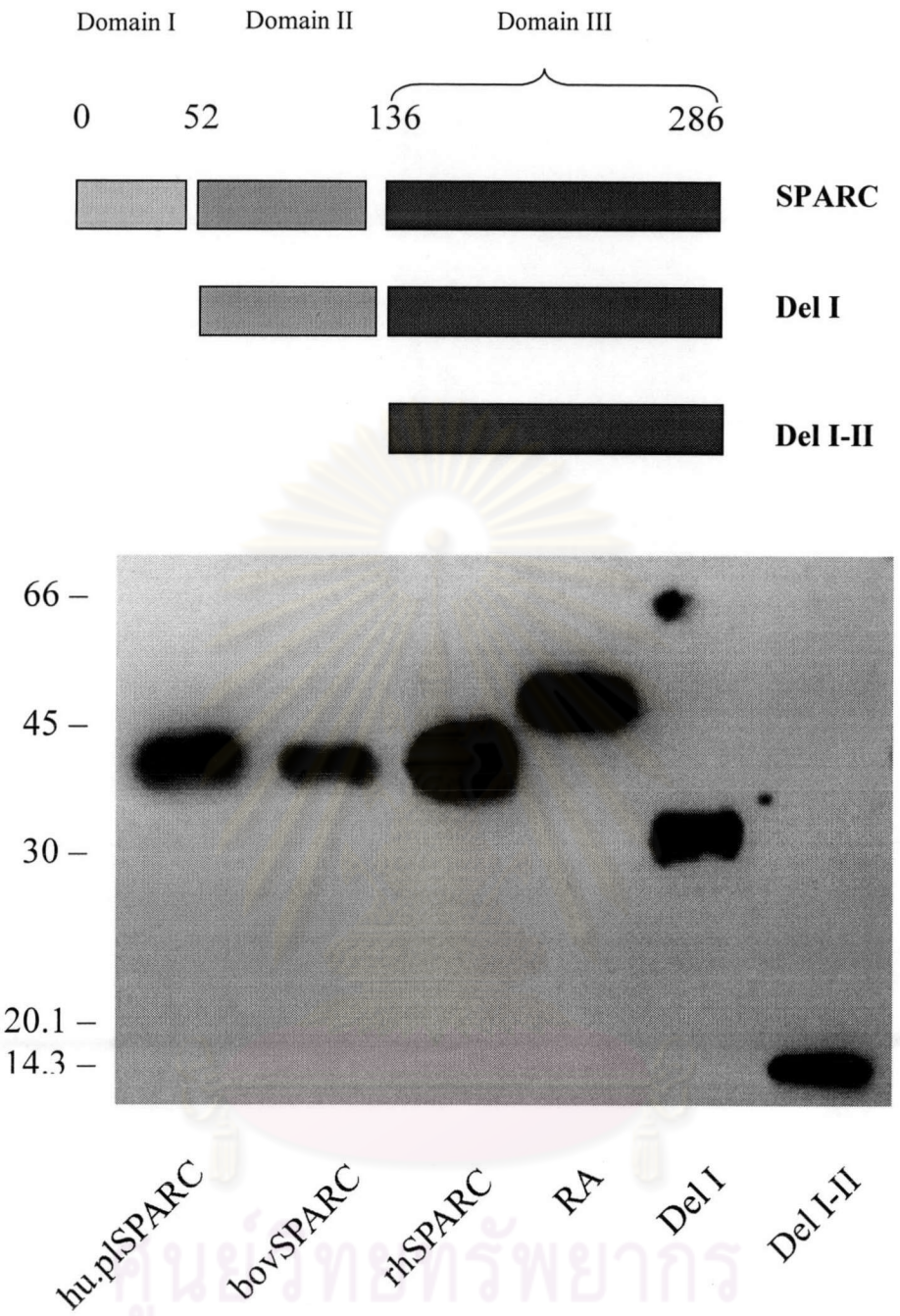

Figure 2.2:

A. A diagram showing SPARC and the deletion mutants used.

B. Western analysis of SPARC and mutants; 50 ng of protein were loaded in each lane.

Previous studies have reported that platelet SPARC ran slightly (around 3 kDa) slower than bone SPARC when analyzed by SDS-PAGE, due to the presence of N-glycosylation, which also reduces the binding capacity of platelet SPARC to collagen type V [13, 15, 16]. However, those differences could not be seen in this study.

We treated BT-549 cells with the SPARC preparations for 72 hours and examined levels of MMP-2 activation and TIMP-2 in the conditioned medium. SPARC induction of MMP-2 activation was not as strong as that seen by collagen or Con A treatment (data not shown), which also increase MT1-MMP levels [135, 158]. Also, MMP-2 activation was not seen with all SPARC preparations that we used. As shown in figure 2.3 panel A, hu.plSPARC, bovSPARC and LN81 showed a strong and reproducible effect on MMP-2 activation. rhSPARC showed a slight increase in activation while rmSPARC had no effect. A slight inhibition could be seen with pmSPARC. However, when we looked at TIMP-2 levels in conditioned media by Western blot, all full length preparations markedly reduced the levels of soluble TIMP-2 (figure 2.3 panel B). The pmSPARC and rmSPARC, which had no detectable effect on MMP-2 activation, showed potent TIMP-2 reduction to a similar degree as the other preparations.

Figure 2.3 also demonstrates the surprising finding that clone no.67-4H11 Fuji anti-TIMP-2 antibody cross-reacted with SPARC, as seen in all treatment lanes as a major band around 43 kDa. In other experiments (data not shown), this TIMP-2 antibody did not cross-react with Del I or Del I-II, but did cross-react with RA, indicating the cross-reaction was with domain I of the SPARC molecule (figure 2.4). Interestingly, this cross-reaction also occurred with mouse SPARC, which has a different sequence in domain I to human and bovine.

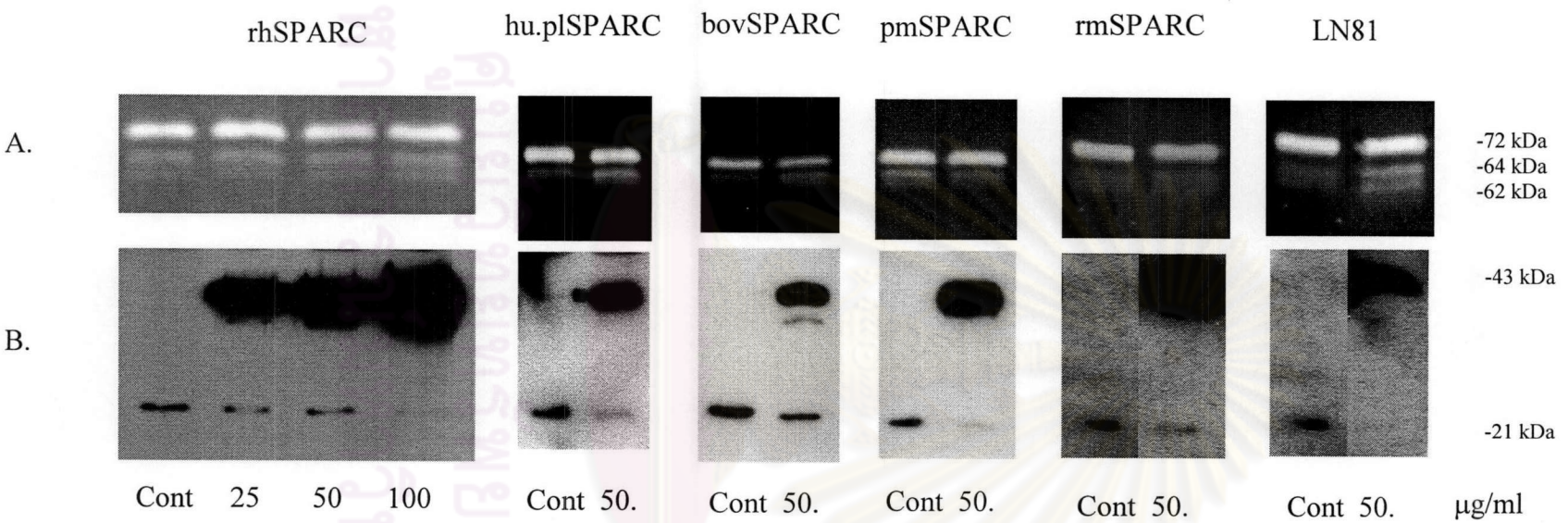

Figure 2.3: Effects of different preparations of SPARC on MMP-2 activation and levels of TIMP-2 in conditioned media. Cells (8,000) were plated overnight in a 96-well plate, changed to DMEM-BSA (100 µl/well) and treated with 25, 50 and 100 µg/ml of SPARC or the vehicle control (Cont). 72-hour conditioned media were collected and subjected to gelatin zymography (A) and TIMP-2 Western analysis (B).

Effects of SPARC peptide and deletion mutants on MMP-2 activation and TIMP-2 levels

Different SPARC deletion mutants were used to further characterize these effects. We also synthesized the 24 residue human SPARC peptide 1.1 [71], which represents part of the acidic amino terminal domain I shown previously to bind hydroxyapatite [69] and to stimulate MMP-2 activation [36]. Mutant delta I (Del I) lacks domain I but contains both the follistatin-like (FS) and the EC domain (residues 53-286), while mutant delta I-II (Del I-II) lacks both domain I and II, and contains only the calcium binding EC domain (residues 136-286). We also tested rhSPARC which has been reduced and alkylated (RA), such structures and sizes of these mutants. The RA preparation ran slightly slower that only domain I is expected to be functional. Shown in figure 2.2 are the domain structures and sizes of these mutants.

Human peptide 1.1 showed strong MMP-2 activation and TIMP-2 reduction in the conditioned medium, indicating the importance of domain I (figure 2.4), and confirming our previous report of bovine peptide 1.1 on this effect [36]. Furthermore, Del I and Del I-II had no effect on MMP-2 activation or TIMP-2 reduction and the RA preparation, in which only domain I is functional, stimulated MMP-2 activation and caused reduced TIMP-2 levels. These data are consistent in their implication of domain I in the reduction of TIMP-2.

Effects of SPARC on MT1-MMP and TIMP-2 mRNA

By Northern analysis, our previous study [36] showed that SPARC had no effect of MT1-MMP and TIMP-2 levels. Here, we confirmed those results using real-time quantitative RT-PCR. With this method, the PCR cycle at which logarithmic growth in the amount of the product is detected (CT value) is directly proportional to

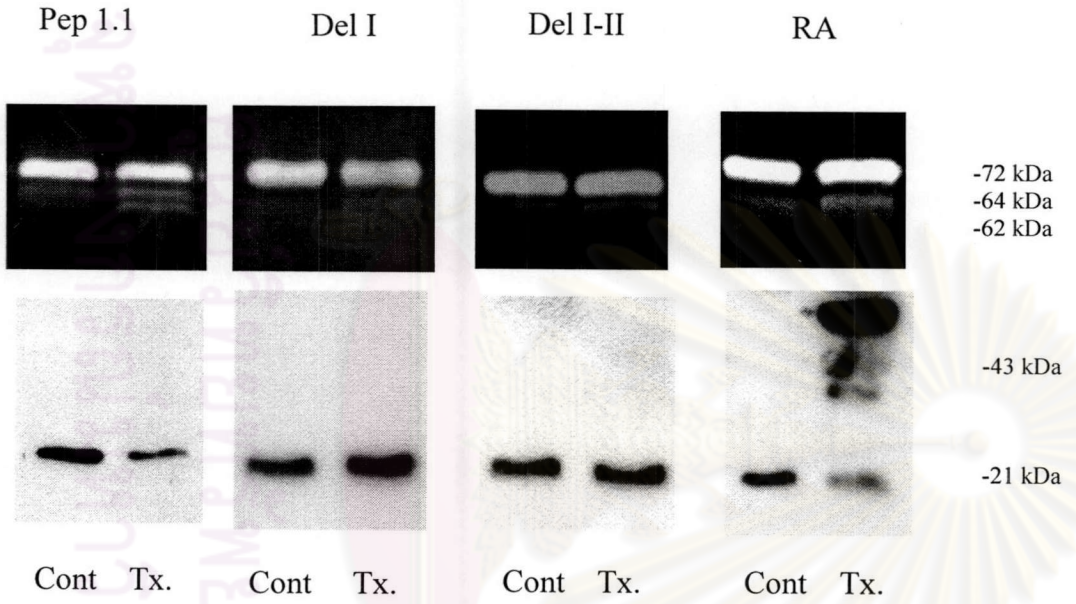

Figure 2.4: Effects of SPARC peptide and deletion mutants on MMP-2 activation and levels of TIMP-2 in conditioned media. Cells (8,000) were plated overnight in a 96-well plate, changed to DMEM-BSA (100 µl/well) and treated with 0.1 mM peptide 1.1 or 50 µg/ml SPARC mutants. 72-hour conditioned media were collected and subjected to gelatin zymography (A) and TIMP-2 Western analysis (B).

		MT1-MMP	TIMP-2	L-32
rhSPARC	Control	33.89/ 33.81	31.95/ 32.06	23.62/ 24.16
	Tx	34.05/ 33.84	32.79/ 32.17	23.50/ 24.00
SPARC mutants	Control	33.29/ 33.39	37.71/ 37.73	29.59/ 29.81
	Del I	32.99/ 32.96	37.48/ 37.90	29.18/ 29.06
	Del I-II	31.88/ 33.27	37.45/ 37.80	29.08/ 28.62

Table 1: Real-time PCR results for MT1-MMP, TIMP-2 and L-32

The data is shown as CT value. These results are from one representative of 3 independent experiments, all of which showed similar trend.

the amount of target present [196, 197]. As shown in Table 1, treatment with rhSPARC, Del I or Del I-II at 50 μg/ml had no effect on the CT value detected for both MT1-MMP and TIMP-2. In contrast, HEK293 cells transiently transfected with with TIMP-2 showed a pronounced reduction in CT value compared to vector alone (data not shown). This confirmed that SPARC had no effect on MT1-MMP or TIMP-2 mRNA levels.

Effects of SPARC on cell-associated TIMP-2

We hypothesised that the reduced levels of TIMP-2 in the conditioned media may be due to increased binding to the cell surface, and compared TIMP-2 levels in cell lysates from SPARC-treated and -untreated cultures. We found that TIMP-2 was mainly present in the conditioned medium under both conditions. Western analysis showed only small amounts of TIMP-2 in the cell lysate, and no apparent difference between SPARC untreated cells and those treated with hu.plSPARC, bovSPARC and pmSPARC (figure 2.5).

Effects of anti-TIMP-2 antibody on MMP-2 activation

Given the imperfect correlation between SPARC-reduced TIMP-2 and MMP-2 activities, we tried to neutralise secreted TIMP-2 using two different anti-TIMP-2 antibodies, as described in Experimental procedures. Antibody concentrations of 0.5, 1, 5 and 10 μg/ml bound TIMP-2 in the culture medium in a concentration-dependent manner, as indicated by the reduction in TIMP-2 levels after the antibodies were removed on protein A Sepharose beads (figure 2.6B). Addition of 1 and 5 μg/ml of each antibody slightly increased MMP-2 activation. Again, MMP-2 activation induced by antibody neutralization of TIMP-2 was not as strong as that seen with

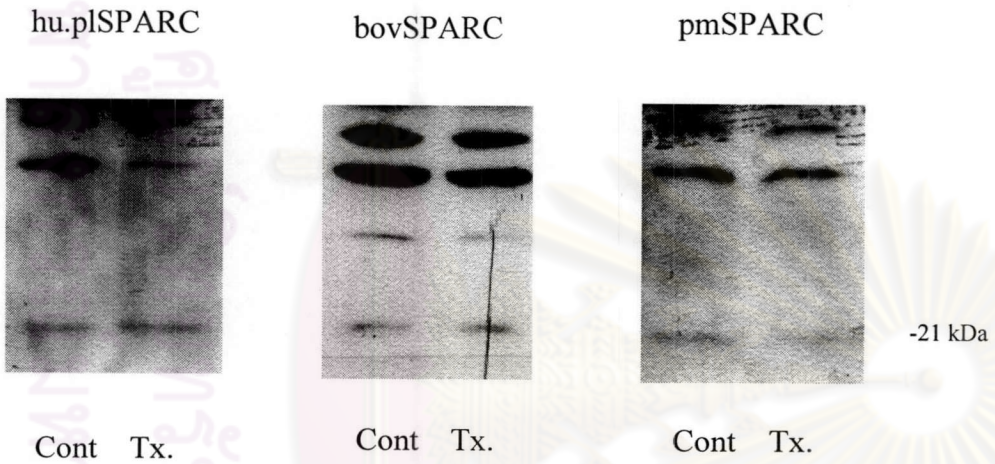

Figure 2.5: Effects of SPARC on cell-associated TIMP-2 levels. Cells (150,000) were plated overnight in a 6-well plate, changed to DMEM-BSA (1 ml/well) and treated with 50 μg/ml SPARC. After 72 hours, cell lysates were extracted in 150μl RIPA with proteinase inhibitors. TIMP-2 Western analysis was performed using 30 μg of protein in each lane.

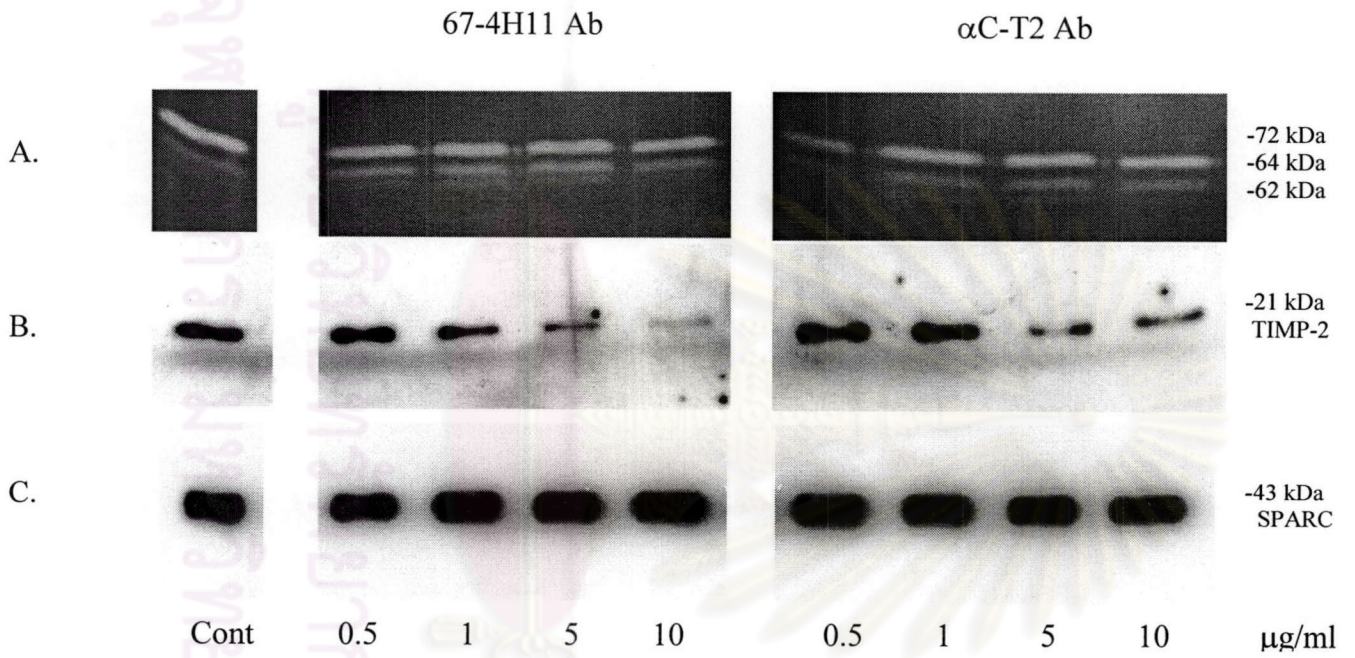

Figure 2.6: Effects of anti-TIMP-2 antibody on MMP-2 activation. Cells (8,000) were plated overnight in a 96-well plate, changed to DMEM-BSA (100 μl/well) and treated with 0, 0.5, 1, 5, 10 μg/ml of each anti-TIMP-2 antibody (67-4H11 and αC-T2Ab). After 72 hours, conditioned media were collected. The antibody was removed by protein A sepharose beads, and the resultant supernatant subjected to gelatin zymogram (A) and TIMP-2 Western analysis (B).

collagen or Con A (data not shown). The level of activation was not stimulated by addition of 10 μg/ml of either antibody presumably because the free TIMP-2 concentrations became too low to support the tri-molecular complex. We also compared the MMP-2 activation pattern in the conditioned medium before and after removal of the anti-TIMP-2 :TIMP-2 complexes by protein A Sepharose, and saw no difference by zymogram (data not shown).

BT-549 cells produce endogenous SPARC [36] and since we found that the Fuji anti-TIMP-2 antibody could cross-react with SPARC in Western analysis, we wanted to make sure that we were not also neutralising the SPARC in these studies. Neither TIMP-2 antibody was able to reduce the levels of endogenous SPARC in the conditioned media when removed with protein A Sepharose (figure 2.6C).

Discussion

SPARC was first purified as a major non-collagenous component of bovine bone [3], and its biological significance was initially focussed on the regulation of bone mineralization. Later studies showed that SPARC is also present in many non-mineralized tissues and in platelets [18-20]. The SPARC gene is highly conserved among vertebrate species, suggesting that the protein has an important physiological role [6]. Our particular interest here is its effect on the TIMP-2: MMP-2 axis.

Many studies have emphasised the important role of TIMP-2 in MMP-2 activation. TIMP-2 binds MMP-2 in a 1:1 ratio, associating with either the catalytic site of the mature enzyme or with the carboxy terminal domain of proMMP-2 [105]. Therefore TIMP-2 can prevent both the activation of the zymogen and the catalytic function of the mature enzyme. However, TIMP-2 is required in the MMP-2 activation process to tether MMP-2 to MT1-MMP at the cell surface [99, 198]. This

delicate balance of TIMP-2 activities regulates net activity of MMP-2, and the simplistic expectation that malignant tumours would have increased MMP expression accompanied by decreased TIMP is not often met [106].

Our previous study [36] using rhSPARC produced in *Escherichia coli* [191] and the synthetic bovine amino-terminal peptide 1.1 showed that each could induce exogenous MMP-2 activation by BT-549 and MDA-MB-231 breast cancer cell lines. A reduction in the level of TIMP-2 in the conditioned medium was also seen. The question still remained as to whether these two effects were interdependent, and whether the TIMP-2 reduction was a cause or an effect of the observed MMP-2 activation.

In the present study, we used various preparations of human, bovine and murine SPARC. Human and bovine SPARC show 99% amino acid sequence identity [9], while human and mouse show 93% [10]. The major difference between human and mouse SPARC is in the amino terminus, located specifically between amino acid residues 5-23 [9], so called peptide 1.1 [71]. However, while considerable divergence exists in this region, the character of the amino acids does not change substantially with the substituted amino acids being primarily acidic in nature [9]. We also compared preparations of SPARC derived from bovine bone or human platelets. A previous study using automated Edman degradation showed identity between the amino terminal sequence of platelet and bone SPARC [13]. However, they were reported to be structurally distinct in a region of the molecule at a distance from the amino terminus, and functionally distinct in terms of glycosylation and collagen binding capacity [15].

All of the intact (full length) SPARC preparations used in this study were able to reduce soluble TIMP-2 levels indicating that the requirements for this are

conserved between species. We went on to examine further the domains responsible for both TIMP-2 reduction and MMP-2 activation with deletion mutants of human SPARC produced in mammalian cells (HEK293). The lack of TIMP-2 reduction with forms of SPARC lacking domain I was well supported by the ability of RA, which has an intact domain 1 but disrupted domains II and III, to cause TIMP-2 reduction. These are also consistent with our previous and current observations that peptide 1.1 caused TIMP-2 reduction, and collectively these data support an important role for the peptide 1.1 region in this process. This domain is considered one of two putative hydroxyapatite-binding regions [69], is highly acidic, and inhibits attachment of endothelial cells and fibroblasts [71]. The mechanism through which SPARC reduces soluble TIMP-2 levels is still unclear. It appeared not to be through reduction in TIMP-2 mRNA levels, as shown by RT-PCR in this study and Northern analysis in our previous study [36]. Also, reduction of soluble TIMP-2 did not appear to be a consequence of increased binding of TIMP-2 to the cell surface, as we were unable to detect changes in TIMP-2 levels by Western analysis of cell lysates. Since the majority of TIMP-2 is present in the media, and only a faint band could be detected in the cell lysates, the question could arise as to whether the technique was sensitive enough to detect changes. However, when considering the ratio of media to lysate, any reduction in media should cause a more dramatic increase in cell lysate by a factor of 8.

Reduced levels of TIMP-2 have been associated with enhanced MMP-2 activation in a number of systems. Ailenberg and Silverman [131] showed that the cytochalasin D enhanced MT1-MMP mRNA and MMP-2 activation of mesangial cells was accompanied by a reduction of soluble TIMP-2 but no change in TIMP-2 mRNA levels. They suggested that soluble TIMP-2 was being reduced by

sequestration to the MT1-MMP on the plasma membrane, forming a trimolecular complex. Similar observations were made in a study in which MT1-MMP was transfected into MCF-7 breast carcinoma, HT-1080 fibrosarcoma and U251.3 glioma cell lines, which resulted in reduction of soluble TIMP-2 and increased in binding to the cell surface [125]. This appears not to be the case here in our studies with SPARC, as we did not detect any effects of SPARC on MT1-MMP levels by RT-PCR in this study, or by Northern analysis in our previous study [36] and also did not see increased cell–associated TIMP-2 by Western analysis. TIMP-2 could be lost from the culture medium through increased degradation. Indeed, in HT1080 and MT1-MMP transfected A2058 cells Maquoi *et al.* [199] showed that TIMP-2 bound to the cell surface through MT1-MMP, was rapidly internalized and degraded in intracellular organelles.

Although all full-length SPARC preparations could reduce soluble TIMP-2, not all also induced MMP-2 activation. Levels of MMP-2 activation were variable, with strong activation seen in hu.plSPARC, bovSPARC and LN81, no effect with rmSPARC, and even a slight inhibition with pmSPARC. These data indicate that the reduction in soluble TIMP-2 was not a consequence of MMP-2 activation in these scenarios. Furthermore, antibody-neutralization of soluble TIMP-2 had a biphasic effect on MMP-2 activation, with stimulation only at intermediate levels. Since TIMP-2 acts as both inhibitor and activator for MMP-2 [100], its optimal level is required for the MMP-2 activation process to proceed, consistent with our results.

In this study using the BT-549 breast cancer cell line, we adjusted the levels of TIMP-2 both by adding exogenous protein, and also neutralization with antibody. We found that this cell already had relatively high levels of TIMP-2 and that reduction through antibody addition helped the MMP-2 activation to proceed, and that addition

of further TIMP-2 immediately inhibited this process. We generally found that MMP-2 activation induced by adjustment of TIMP-2 levels was not as strong as that induced by other means that also affect MT1-MMP levels, such as treatment with collagen or Con A [135, 158]. However, these treatments both stimulate the expression of MT1-MMP and regulate the activity of pre-existing MT1-MMP whereas SPARC appears only to affect the latter. This, and the delicate balance required in TIMP-2 levels may explain the inconsistencies between MMP-2 activation effects of SPARC and their ability to reduce soluble TIMP-2. It is also possible that the SPARC effects on the TIMP-2 reduction and MMP-2 activation are independent events, and that the latter is due to molecular aspects of SPARC which vary among preparations. Given the capacity of SPARC to reduce TIMP-2, and the activation seen with anti-TIMP-2 treatment, it seems very likely that whatever activation we do see with different SPARC preparations is due to the reduced TIMP-2, but we have no proof for this. Furthermore, the consistent results seen on both TIMP-2 and MMP-2 activation with peptide 1.1 would localize both effects to this relatively small region of SPARC.

In conclusion, this study showed that human, bovine and mouse SPARC from a variety of sources are able to reduce soluble TIMP-2 levels. Domain I of the SPARC molecule is responsible for this activity, and specifically peptide 1.1. TIMP-2 reduction by SPARC is not due to changes in TIMP-2 mRNA or binding to the cell surface, and is not a consequence of SPARC induce MMP-2 activation and so may be due to increased degradation. Not all SPARC preparations could induce MMP-2 activation, despite pronounced reduction in TIMP-2; perhaps they reduce TIMP-2 to a suboptimal level. Further study is required on how SPARC reduces soluble TIMP-2, and the relationship between reduction of soluble TIMP-2 by SPARC and SPARC-induced MMP-2 activation.