Online supplement for the manuscript:

Matrix Metalloproteinase II Activation of TGF-β1 and TβRII Signaling within the Aged Arterial Wall

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Materials and Methods

Animals

Male Fisher 344 crossbred Brown Norway rats (FXBN), 8-month-old (8mo) and 30-month-old (30mo), were obtained from the National Institute on Aging Contract Colonies (Harlan Sprague Dawley, Inc, Indianapolis, IN). The animal protocol used was approved by the Institutional Animal Care and Use Committee of the Gerontology Research Center and complied with the guide for the care and use of laboratory animals (NIH publication No. 3040-2, revised 1999).

Ex vivo Organ Culture

Aortic rings were explanted and cultured as previously described (1). Briefly, after dissection of adventitial tissue, aortic segments were placed in 6-well dishes containing DMEM+F12 (1:1) (GIBCO BRL) and antibiotics (penicillin 100 U/mL, streptomycin 100 mg/mL) and supplemented with transferrin 5µg/mL, and insulin 5µg/mL. The aortic rings from young rats were then maintained in a tissue culture incubator at 37°C and exposed to activated MMP-2 (0, 100, and 400ng/ml). Active MMP-2 enzyme (Cat# PF023) was purchased from Oncogen Research Products. Human recombinant MMP-2 was purified from mammalian cells. The pro-enzyme form of MMP-2 was activated following the
manufacturer instructions using organomercurial compound, 4-aminophenyl mercuric acetate (APMA).

Aortic rings from old animals were exposed to MMP inhibitors: GM6001 (0, 100, 1000, and 10000 ng/ml), or recombinant human Tissue Inhibitor of Metalloproteinase-2 (rhTIMP-2) (0, 100, 500, and 1000 ng/ml). The vascular segments were frozen in LN2 and were stored at -80°C until use for protein analysis by gelatin zymography and Western Blotting.

**VSMC Isolation and Culture**

VSMC were enzymatically isolated as previously described (2). Briefly, F344XBN rat thoracic aortae were rinsed in Hanks balanced salt solution (HBSS) containing 50µg/mL penicillin, 50µg/mL streptomycin and 0.25µg/mL amphotericin B (Gibco). After digestion for 30 min in 2mg/mL collagenase I solution (Worthington Biomedical, Freehold, New Jersey) at 37°C, the adventitia and intima were removed from the vessel media layer, which was placed overnight in complete medium (DMEM plus 10% FCS). On day 2 the vascular media was further digested with 2mg/mL collagenase II/0.5mg/mL elastase (Sigma) for 1 hour at 37°C, and the isolated cells were washed and plated in complete medium. In all cases, >95% of cells stained positive for α-smooth muscle actin (α-SMA).

Early passage (p3-p5) VSMC were cultured with MMP-2 (100 ng/ml), GM 6001 (6000 ng/ml), rhTIMP-2 (500 ng/ml), or PAI-1 (0, 10, and 100ng/ml), for 24h, in 2.5% FBS DMEM.
**Real-Time PCR analysis**

Real-time PCR was performed using the SYBRGreen PCR based protocol in a 384-well plate format (Applied Biosystems, Foster City, CA) as previously described (2). Messenger RNA was extracted from the thoracic aortae of four individual young and old rats using the TRizol system (Life Technologies, Rockville, Md). RNA (500ng) was reverse transcribed for 30 minutes at 48°C using random hexonucleotides according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). TGF-β1 and TβRII transcript levels were analyzed by Real-time PCR using the SYBRGreen PCR based protocol. Gene-specific forward and reverse primers for TGF-β1 (forward: GACGGAATACAGGGCTTTCG, reverse: CCTCGACGTTTGGGACTGAT, amplified length: 101bp) and TβRII (forward: CAGCTGTGCAAGTTTGCGA, reverse: TTCTGCGCCTTCTCACAGAT, amplified length: 107bp) have been designed using Primer Express software 1.5 (Applied Biosystems, Foster City, CA), and purchased by Invitrogen Life Technologies (Carlsbad, CA). Each sample has been tested in quadruplicate. The reaction conditions were: 10 min at 95°C (one cycle), and 15 sec 95°C, 20 sec 60°C, and 30 sec 72°C (40 cycles). Gene-specific PCR products were continuously measured by means of an ABI PRISM, 7900 HT Sequence Detection System (PE Applied Biosystem, Norwalk, CT) and the PCR product sizes were verified by agarose gel electrophoresis. Data are expressed as the mean quantity, calculated as follows: quantity=10-(Ct-Y intercept)/slope value), where Ct represent the threshold cycle value. Results are expressed as a ratio of signals corresponding to the gene of interest and the rRNA 18s levels, which did not change with age.
Co-immunoprecipitation and Western Blot analysis

Immediately after harvesting, F344XBN rat thoracic aortae were rinsed in 1X PBS, frozen in liquid nitrogen and pulverized in the presence of 200 µL of ice-cold lysis buffer (LB: 20 mM Tris pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Triton X-100, 1µg/mL leupeptin; 1 mmol/L PMSF). Lysates were centrifuged at 10,000 rpm for 30 min and 200µg of the supernatants were incubated (30 min, 4°C, rolling) with 20µl of a 50% Protein A-Sepharose slurry (Sigma) for preclearing. The post-spin supernatant was then incubated overnight at 4°C with rabbit anti-TGF-β1 or normal rabbit IgG (Santa Cruz). Protein A Sepharose beads (20µl of the 50% slurry) were then added for 4 hours with continuous agitation at 4°C. Immunoprecipitates were washed in LB buffer, suspended in 20µl of SDS-loading buffer, boiled and run on 12% SDS-PAGE gels followed by Western blotting with an anti-MMP-2 antibody (Chemicon).

For Western Blotting, fifteen µg of whole cell lysates were resolved by SDS-PAGE and transferred onto PVDF membrane (Immobilon). The transferred membranes were incubated in PBS containing primary antibodies (Table) at 4°C for 24 hours. HRP-conjugated IgG (Amersham Pharmacia Biotech, Buckinghamshire, GB) were used as secondary antibodies and detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL). The average densitometric analysis of the bands was obtained on the protein extracts from the aortae of four rats of each age group or from VSMC at least three independent experiments. Beta-actin immunoblotting has been used as a protein loading control since its expression does not change with age.
**In situ Zymography**

To localize net *in situ* gelatinolytic activity of MMPs by zymography, FITC-labeled DQ gelatin intra-molecularly quenched (Molecular Probes, Eugene, OR) was used as a substrate. Fresh aortae were collected and rinsed in cold PBS to remove outside connective tissue. The aortae were then immersed in ornithine carbamyl transferase (OCT) (Tissue-Tek, Torrance, CA) and quick-frozen into a block in dry ice. The aortae in the OCT blocks were cut into 10 μm sections using a cryostat (Leica, Wetzler, Germany) and collected sequentially. *In situ* zymography was performed by modification of a combination of two methods previously described (3, 4). FITC-labeled DQ gelatin (1 mg/mL) was mixed (1:1) with 1% agarose, and melted in reaction buffer (0.05 mol/L Tris-HCl, 0.15 M NaCl, 5 mM CaCl₂, and 0.2 mmol/L NaN₃, pH 7.6). The liquid mixture was spread on pre-warmed glass slides by a maneuver similar to that used to produce blood smears, and the film was allowed to gel at room temperature. Frozen sections of unfixed tissue were cut and applied to the top of the substrate film. A drop of the reaction buffer was added over each tissue section, and a silated coverslip was applied. Slides were incubated in light-protected humidified chambers at 37°C for 48 hours. At the end of the incubation period and without fixation or washes, lysis of the substrate was assessed by examination under fluorescence microscopy. As a negative control for *in situ* zymography, the frozen sections were processed as above but without the FITC-labeled DQ gelatin, and 3.8μg/mL of an antibody against MMP2 was added to the reaction to inhibit metalloproteinase.
**Immunohistochemistry and Immunofluorescence**

Immunostaining was performed according to the protocols provided by the manufacturer (Dako Corp.CA). The negative control was stained with serum (no primary antibody).

The source and characteristics of primary antibodies used were listed in the table.

**Table. Primary Antibodies.**

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Species</th>
<th>Immunostaining</th>
<th>Western Blot</th>
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**In vitro activation assay for LAP-TGF-β1**

The *in vitro* activation of LAP-TGF-β1 by MMP2 was performed according to the modified method previously described (5-7). Briefly, LAP-TGF-β1 (5ng/ml, R&D
Systems Inc.) was cleaved by 100ng/ml activated MMP-2 (Cat# PF023)) with 50, and 500 ng/ml rhTIMP2 (R&D Systems Inc.) in DMEM containing 0.1% BSA at 37E for 30 minutes. The reactions were stopped with 20mM EDTA. The activated TGF-β1 was determined by Western blot analysis.

Statistical Analysis
All results are expressed as the mean ± SEM. Statistical analysis was performed via a T-test when two groups were analyzed, or via an ANOVA, followed by a Bonferroni post hoc test for multiple comparisons. A p value of <0.05 was taken as statistically significant.

References


Figure legends

**Figure I. LTBP Immunostaining.** Immunofluorescence staining, detected with FITC (green color), for LTBP and immunohistochemical staining, detected with DAB (brown color), for LTBP (inset). L=lumen; M=media. Original magnification: X400.

**Figure II. TGF-β1 protein staining co-localizes with CD31 and α-SMA staining**. Double immunofluorescence staining of aged rat aortic paraffin sections. TGF-β1 is detected with TRIC (middle panels, red color), CD31 and α-SMA are detected with FITC (left panels, green color). The merged images (right panels) indicate that TGF-β1 staining co-localizes with that of CD31 and α-SMA (yellow color). L=lumen; M=media. Original magnification: X 400.

**Figure III. Lymphocyte and macrophage cell detection.** Immunostaining with antibodies against CD45, a lymphocyte marker, and CD68, a macrophage marker shows the absence of these cell types in the intima and media (upper panels) and the occasional presence in the adventitia (middle panels) within the old rat aorta. Same strain rat liver is shown as an immunostaining positive control (brown color, lower panels). L=lumen; M=media, A=adventitia. Original magnification: X 400.

**Figure IV. TβRII and SMAD expression increases within the aged rat aorta.** A. Representative agarose gel of TβRII RT-QPCR. B. Average data. C. Representative Western Blot for TβRII in young and old rat aortae. D. Average data. *p<0.01, old versus
young. E. Representative Western Blots for SMAD2/3 (top panel), SMAD4 (middle panel), and SMAD7 (bottom panel) of young and old rat aortae. F. Average densitometrical analysis. *p <0.05, young versus old

**Figure V.** Exposure of MMP-2 to young aortic rings increases ring-associated MMP-2 activity. Representative zymogram.

**Figure VI.** MMP-2 effect on matrix production. A. Western Blots for p-SMAD2/3, Coll I, Coll III, and FN in young rat aortic rings exposed to activated MMP-2 for 72 hours and old untreated rings. Middle panels: average data. *p <0.01 versus untreated young aortic rings (left panels). Right panels show Western blots for Coll I, Coll III, and FN correspondingly of old arterial rings treated with rhTIMP-2 for 72 hours. B. Western blots for Coll I, Coll III, and FN in VSMC treated with or without MMP-2, rhTIMP-2, or GM6001. Right panels: average data. *p <0.05, versus young untreated VSMC.

**Figure VII.** PAI-1 reduces both MMP-2 and TGF-β1 activity in old VSMC. A. Representative Western blots for PAI-1 from young and old aortae and average data. B. Representative zymogram (upper panel) and Western blots for TGF-β1 (middle panel) and β-actin (lower panel).
Figure IV
Figure V
Figure VII