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Decoupled Syndecan 1 mRNA and Protein Expression Is Differentially Regulated by Angiotensin II in Macrophages

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Abstract

It has been established that syndecan-1 is an important modulator of events relevant to acute tissue repair and chronic injury responses. The current studies were designed to examine syndecan-1 expression during atherosclerotic lesion formation and whether angiotensin II influences syndecan-1 expression in macrophages. ApoE knockout mice maintained on an atherogenic diet were treated for 8 weeks with an infusion of angiotensin II to induce atherosclerosis. Immunohistochemistry was employed to characterize the expression of syndecan-1 in atherosclerotic lesions. Quantitative real-time PCR (QRT-PCR) was used to define the role of angiotensin II and responsible signaling pathways involved syndecan-1 expression in RAW264.7 murine macrophages. Protein expression and shedding were characterized by fluorescence activated cell sorting (FACS) and slot blot analysis. Syndecan-1 was abundantly expressed in macrophages located within early atherosclerotic lesions. Accordingly, we hypothesized that angiotensin II regulates syndecan-1 expression in macrophages. A time- and dose-dependent study was performed in RAW264.7 macrophages. QRT-PCR demonstrated maximum syndecan-1 mRNA up-regulation at 6 h after 500 nM AgII stimulation (threefold; $P < 0.05$). Through administration of specific inhibitors, we established that ERK/MAPK, PI3K and JNK signaling pathways mediated this effect. FACS and slot blot analyses demonstrated that cAMP induced posttranscriptional syndecan-1 protein expression in a dose-dependent manner with or without initial angiotensin II stimulation. In particular, angiotensin II induced an increase in cell surface syndecan-1 (mean fluorescence intensity: 147 ± 5.7 vs. 176 ± 4.8 ; $P < 0.05$; $n = 3$) and accelerated syndecan-1 shedding. Angiotensin II is a potent regulator of syndecan-1 expression in atherosclerotic lesions via a specific effect on macrophages that is mediated by ERK/MAPK, PI3K, and JNK signaling pathways.

Monocytes are recruited to the vascular wall during the process of atherogenesis where they differentiate into macrophages and acquire the capacity to synthesize an array of heparin-binding cytokines, chemokine, and growth factors. Significantly, the biological activity of many of these macrophage-derived factors is regulated, both positively and negatively, by interactions with heparan sulfate proteoglycans (HSPG) both on the cell surface and shed into the extracellular matrix. In this regard, members of the syndecan family constitute a major source of cell surface heparan sulfate and, in particular, syndecan-1 has been identified as the predominant source of surface HSPGs on murine macrophages (Yeaman and Rapraeger, 1993a,b; Kim et al., 1994; Bernfield et al., 1999). Several investigations have noted that syndecan-1 expression on vascular smooth muscle cells, as well as other mesenchymal cells,

may be regulated by a variety of soluble growth factors, such as FGF-2, PDGF, and TGF- β 1, released at sites of acute or chronic injury (Bansal et al., 1996; Cizmeci-Smith et al., 1997; Worapamorn et al., 2001; Li et al., 2002). However, little detail exists regarding those events that control syndecan-1 expression and shedding in macrophages.

Substantial evidence now supports the notion that locally generated angiotensin II may exert significant autocrine and paracrine effects within the vascular wall that may promote atherosclerosis. For example, angiotensin II increases macrophage lipooxygenase activity with concomitant generation of pro-atherogenic oxidized LDL, thereby enhancing monocyte recruitment into the arterial wall (Scheidegger et al., 1997). Angiotensin II also stimulates macrophage HMG CoA reductase gene expression, which leads to increased biosynthesis of cholesterol, as well as uptake of oxidized LDL and, presumably foam cell formation (Keidar et al., 1995, 1999, 2001; Kaplan et al., 2002). Kranzhofer et al. (1999) have also noted that angiotensin II activates the proinflammatory transcription factor nuclear factor-kappa B in human monocytes.

In this investigation, we report that angiotensin II induces syndecan-1 expression in atherosclerotic lesions via a specific effect on macrophages. Furthermore, correlative in vitro studies reveals that angiotensin II upregulates macrophage syndecan-1 mRNA and protein expression by a direct effect on ERK/MAPK, PI3K, and JNK signaling pathways. Given the potential role of syndecan-1 as a modulator of local growth and inflammatory activity, these data suggest that angiotensin II may contribute to the development of atherogenic lesions, at least in part, through the induction of macrophage syndecan-1.

Materials and Methods

Animal model

All experimental procedures were approved by the Institutional Animal Care and Use Committee and were in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH). Eight-week-old male ApoE-deficient mice (C57BL/6 background), purchased from the Jackson Laboratory (Bar Harbor, ME), received an atherogenic diet (Research Diets, New Brunswick, NJ) with subcutaneous angiotensin II infusion at a dose of 0.75 mg/kg/day (Sigma, St. Louis, MI). The mice were fed ad libitum and had free access to water. After 8 weeks, mice were euthanized by CO₂ inhalation, aortic tissues were harvested for histological analysis, as described below.

Implantation of mini-osmotic pumps

Osmotic minipumps (Model 2004, Alza Scientific Products, Mountain View, CA), filled with angiotensin II solution, were implanted into the subcutaneous space. The dosage of angiotensin II for each mouse was determined individually to ensure the delivery of 0.75 mg/kg/day. Blood pressures were 103.9 $\pm$ 1.5 mmHg (n=60) at baseline and 181.3 $\pm$ 14.8 mmHg (n=6) at 8 weeks.

Histology and immunohistochemistry

After animals were euthanized, the heart and aorta were pressure-perfused with 0.9% NaCl solution. Aortic tissue was harvested, embedded in OCT (Tissue-Tek; Miles, Inc., Elkhart, IL), snap-frozen, and 5 μ m transverse cryosections prepared. For histological analysis, sections were fixed in cold acetone and stained with hematoxylin and eosin. For immunohistochemical analysis, sections were fixed in cold acetone, blocked with 10% sera from the species used to generate the secondary antibody.

Immunocytochemical staining was performed to identify syndecan-1 and macrophages. At least 2 slides, containing 15 tissue sections, from each animal were examined for each cell

type. The following reagents were used: goat anti-syndecan 1 antibody (N-18, Santa Cruz Biotechnology, Santa Cruz, CA; 1:50 dilution) and rat anti-mouse Mac3 antibody (BD Pharmingen, San Diego, CA; 0.625 µg/ml) for macrophages. Negative controls with goat or rat IgG were prepared for each specimen. Sections were then incubated with biotinylated secondary antibodies (Vector Labs, Burlingame, CA) followed by streptavidin-alkaline phosphatase (Vector labs, 1:500 dilution). Vector Red substrate kit I (Vector labs) was used as a substrate for alkaline phosphatases. Nuclei were visualized by counterstaining with hematoxylin.

Cell culture

The mouse macrophage RAW264.7 cell line (ATCC; Manassas, VA; TIB-71; passages 2–15) was used in this study. Unless otherwise specified, cells were maintained in Dulbecco's modified Eagle's medium (ATCC) supplemented with 10% fetal bovine serum.

Quantitative real-time RT-PCR (QRT-PCR)

Total RNA from cultured macrophages was extracted (RNeasy kit; Qiagen) and reverse transcription reaction (Advantage RT for PCR kit; CLONTECH, Mountain View, CA) was performed with 1 µg of DNase I (Qiagen)-treated RNA. QRT-PCR was carried out using the ABI thermocycler and the Taqman Gene Expression Assay kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The information for the primers and probes, as well as the reaction conditions can be found at <http://www.appliedbiosystems.com>. Expression levels of target genes were normalized by concurrently measured 18S mRNA levels.

Flow cytometry

Flow cytometry was employed to measure cell surface syndecan-1 expression levels. Samples were immunostained using a three-step protocol with syndecan-1 antibody (SC-7100, Santa Cruz Biotechnology), biotinylated anti-Ig, and PE-conjugated streptavidin. Flow cytometry was carried out with a fluorescent-activated cell scan (FACS) operating with FlowJo software (Becton Dickinson, Mountain View, CA). The total population of viable cells was gated according to the results of BD Via-probe (BD Biosciences Pharmingen, San Jose, CA; Cat: 555815) staining. The fluorescence of cells treated with fluorescent isotype control IgG was evaluated in each experiment to determine the level of background fluorescence of negative cells. Mean fluorescence intensity (MFI) was determined for positively stained cells. As additional control experiments, primary antibody titration (1:50 to 1:200), as well as secondary and/or tertiary antibody staining without primary antibody was performed for samples showing increased syndecan-1 expression to exclude a false positive result.

Slot blot immunoassay

Specificity and quantitation of the slot blot immunoassay for shed syndecan-1 has been described previously (Subramanian et al., 1997; Fitzgerald et al., 2000). In brief, conditioned media were filtered using Ultrafree Durapore Centrifugal Filter Devices (0.2 µm, Millipore, Billerica, MA) and concentrated to a minimum volume. The samples were then diluted in a buffer (0.15 M NaCl buffered to pH 4.5 with 50 mM sodium acetate, and with 0.1% Triton X-100) and applied to cationic polyvinylidene difluoride-based membrane (Immobilon-NY+; Millipore) under mild vacuum in an immunodot apparatus (Bio-Rad Laboratories, Hercules, CA). By acidifying the samples in the above buffer, only highly anionic molecules in the conditioned media, such as proteoglycans, are retained by the cationic Immobilon-NY+ membrane while most proteins are cationic at this pH and pass through the membrane. The membranes were then washed twice with the above buffer. The blocking and hybridization procedures are the same as described previously (Wang et al., 2002b). Syndecan-1 (Clone

281.2; Cat. 553712; BD Pharmingen) antibody was employed for the immunoblots. Membranes were developed using an enhanced chemiluminescence method (Ecl-Luminol kit; Santa Cruz Biotechnology).

Experimental protocols for induction of syndecan 1

The mRNA expression levels of syndecan-1 in cultured RAW264.7 cells were investigated by QRT-PCR at different time points (0–48 h) after angiotensin II stimulation (10–1,000 nmol/L; Sigma). Postconfluent RAW264.7 cells were kept quiescent in OPTI-MEM I (Invitrogen, Carlsbad, CA) for 24 h before angiotensin II administration in serum free medium for the time points indicated. The mediator role of the ERK, JNK, phosphatidylinositol 3-kinase (PI3K), P38, and PKC pathways was evaluated in angiotensin II-treated RAW264.7 cells after 1 h of treatment with the following inhibitors, respectively: U0126 (10 μ mol/L; Biomol International, Plymouth Meeting, PA), SP600125 (20 μ mol/L; Biomol), LY294002 (50 μ mol/L; Sigma), SB203580 (10 μ mol/L; Biomol), and GF109203X (2 μ mol/L; Biosource, Camarillo, CA). The efficacy of pathway blockade at these doses has been previously described (Wang et al., 2002a; Smyth et al., 2006).

For dibutyryl cAMP (dbcAMP) induction of syndecan-1 protein expression, confluent RAW264.7 cells were stimulated by 0–1 mmol/L dbcAMP in serum free medium for 12 h. For the angiotensin II and/or dbcAMP double stimulation experiments, postconfluent RAW264.7 cells were kept quiescent in OPTI-MEM I for 24 h before administration of 500 nmol/L of angiotensin II for 6 h followed by 12 additional hours of 1 mmol/L dbcAMP stimulation in serum free medium.

Statistical analysis

All experiments, including slot blots, were independently repeated at least three times. Comparisons between two groups were analyzed via a Student's *t*-test, and values of $P < 0.05$ were considered to be significant. Results were presented as mean $\pm$ SEM. At least three different samples were analyzed in each experimental group.

Results

Syndecan-1 is abundantly expressed in atherosclerotic lesions

It is well established that angiotensin II is an important mediator of vascular remodeling and lesion formation (Griendling and Alexander, 1997; Griendling et al., 1997; Weiss et al., 2001a,b). Thus, we postulated that the well-described modulation of atherosclerotic lesion formation in response to angiotensin II involves a coordinate regulation of syndecan-1 expression in macrophages. Immunohistochemical staining of the aorta of ApoE^{-/-} mice prior to infusion of angiotensin II revealed that syndecan-4 is constitutively expressed by the smooth muscle cells in the media. In contrast, syndecan-1 was not expressed within the aortic wall of age matched mice that were not subjected to either angiotensin infusion or a high fat diet. After infusion of angiotensin II for 8 weeks, atherosclerotic lesions were noted. Both Mac3 and syndecan-1 positive staining were observed throughout the atherosclerotic plaque (Fig. 1). Colocalization of syndecan-1 staining with infiltrating macrophages was confirmed by double immunofluorescence staining (data not shown).

Angiotensin II upregulates syndecan-1 mRNA expression in cultured macrophages in a time- and dose-dependent manner

A time course (0–48 h) and dose-dependent (0–1,000 nmol/L angiotensin II) study was performed to characterize the effect of angiotensin II on RAW264.7 macrophages in vitro. As depicted in Figure 2, quantitative real-time PCR demonstrated that syndecan-1 is abundantly

expressed in the RAW264.7 macrophages and the maximum syndecan-1 mRNA expression occurs 6 h after 500 nmol/L AgII stimulation (~ 3 fold, $n=3$, $P < 0.05$).

Angiotensin II-induced upregulation of syndecan-1 mRNA occurs via multiple signaling pathways in macrophages

To examine the cellular mechanisms that links angiotensin II to the regulation of the syndecan-1 mRNA expression, we initially assessed the role of ERK/MAPK signaling pathway. QRT-PCR analysis demonstrated that both constitutive and angiotensin II-induced syndecan-1 mRNA expression could be abolished by ERK/MAPK pathway blockade with the pharmacologic MEK inhibitor U0126 (Fig. 3A). Likewise, angiotensin II-induced upregulation of syndecan-1 mRNA could be attenuated by PI3K and JNK blockade by pharmacologic inhibitors LY294002 (Fig. 3B) and SP600125 (Fig. 3C), respectively. Neither p38 (SB203580) or the PKC (GF109203X) inhibitors affected angiotensin II induced syndecan-1 mRNA expression (data not shown).

Syndecan-1 mRNA and protein expression are decoupled in RAW264.7 macrophages

After determining the expression and regulation of syndecan-1 mRNA by angiotensin II, syndecan-1 protein expression in cultured macrophages was characterized after angiotensin II stimulation. Using FACS analysis to characterize cell surface syndecan-1 expression (Fig. 4A,B) and slot blot for syndecan-1 shedding in cell culture supernatants (Fig. 4C), we demonstrated that the syndecan-1 protein expression was similar in angiotensin II-treated and non-treated controls, both of which have low basal levels of expression. These data are consistent with results published by Yeaman and Rapraeger (1993b) who observed that a posttranscriptional block exists for syndecan-1 protein in thioglycollate-elicited peritoneal macrophages. We concluded that syndecan-1 mRNA and protein level expression are decoupled in cultured RAW264.7 macrophages.

dbcAMP is a potent inducer of syndecan-1 protein expression and shedding in RAW264.7 macrophages

Yeaman and Rapraeger (1993b) have observed that dbcAMP, which can raise intracellular cAMP levels, is a potent inducer of syndecan-1 expression in thioglycollate-elicited peritoneal macrophages. Similarly, we noted that dbcAMP can promote the expression and shedding of syndecan-1 in a dose-dependent manner in RAW264.7 cells, as demonstrated by FACS (Fig. 5A,B) and slot blot (Fig. 5C) analyses. Maximum induction occurred at 1 mmol/L dbcAMP after a 12 h incubation period ($n=3$; $P < 0.05$).

Angiotensin II-induced syndecan-1 mRNA accumulation can be translated to protein expression by dbcAMP induction with induction of syndecan-1 shedding

After demonstrating that dbcAMP is a potent inducer of syndecan-1 protein expression, its capacity to induce the translation of angiotensin II-induced syndecan-1 mRNA was explored. After a 24 h quiescence period, postconfluent RAW264.7 cells were incubated in the presence or absence of angiotensin II (500 nmol/L) for 6 h followed by exposure to 1 mmol/L dbcAMP for 12 h. FACS analysis (Fig. 6A) showed that 6 h of angiotensin II stimulation resulted in a small increase in cell surface syndecan-1 protein expression (MFI: 147.11 ± 5.73 vs. 176.86 ± 4.8 ; $n=3$; $P < 0.05$). Whereas by slot blot analysis we observed a significant increase in syndecan-1 shedding (Fig. 6B). These results suggest that angiotensin II-induced syndecan-1 protein expression and shedding requires elevated levels of intracellular cAMP.

Discussion

Although the synthesis of heparan sulfate by macrophages and its roles in regulating lipid uptake have been well documented (Edwards et al., 1995; Seo and St Clair, 1997), how macrophages regulate the bioavailability of growth factors, proteases, chemokines in a manner that may contribute to atherogenesis remains ill-defined. In general terms, the action of heparin-binding growth factors, proteases, and chemokines is known to be regulated by interactions with HSPGs on the cell surface and within the extracellular matrix (Kim et al., 1994; Bernfield et al., 1999). Indeed, macrophages are an important and abundant source of both heparin binding bioactive proteins and HSPGs. We have postulated that macrophage syndecan-1 through its role as a cell surface HSPG may modulate events relevant to acute tissue repair and chronic injury responses, including cell migration and proliferation, cell-substrate interactions, and matrix remodeling. Syndecan-1 can bind and modulate the activity of a diverse group of soluble and insoluble ligands, such as extracellular matrix components (e.g., fibronectin and collagen), growth factors (e.g., FGF, TGF- β , and PDGF), CC and CXC chemokines, and proteases (e.g., MMP-1, 2, 9, cathepsin G, and neutrophil elastase; Bernfield et al., 1999). Although syndecan-1 may modulate cell behavior through its action as a cell surface co-receptor, substantial evidence suggests that accelerated syndecan shedding into the ECM may provides another mechanism for regulating local host responses to tissue injury (Park et al., 2000). All told, it is plausible that chronically accelerated syndecan-1 shedding could potentiate atherosclerotic lesion formation by promoting the generation of a sustained proinflammatory, growth-stimulating environment. To date, the expression of syndecan-1 during the evolution of an atherosclerotic lesion has not been defined. Significantly, mouse models provide a convenient starting point for the study of atherosclerosis and syndecan-1 has been identified as the major cell surface HSPG in murine macrophages (Yeaman and Rapraeger, 1993a,b).

Several lines of evidence now implicate the renin-angiotensin system in the pathogenesis of atherosclerosis (Griendling and Alexander, 1997; Griendling et al., 1997). For example, clinical studies have demonstrated that patients with high renin hypertension are at increased risk of atherosclerosis and myocardial infarction (Meade et al., 1993; Alderman, 1996; Alderman et al., 1997; Yusuf et al., 2000). Likewise, angiotensin II treatment produces a dramatic increase in atherosclerosis in ApoE-deficient animals, even on a regular chow diet, with atherosclerotic lesions characterized by marked macrophage infiltration (Weiss et al., 2001a,b). In turn, in both animal models and clinical trials, administration of angiotensin converting enzyme inhibitors or angiotensin receptor antagonists reduces the development of atherosclerotic lesions, as well as adverse cardiovascular events (Schuh et al., 1993; Kowala et al., 1994; Keidar et al., 1997; Makaritsis et al., 1998). These and other data serve to emphasize that even in the absence of high systemic levels of angiotensin II, locally generated angiotensin II may have a significant impact on the development of atherosclerosis.

Angiotensin II appears to accelerate and worsen atherosclerosis through a multifactorial process. The induction of hypertension with increased wall stresses suggests that mechanical factors may contribute to lesion formation. However, local production angiotensin II may increase NAD(P)H oxidase activity in vascular smooth muscle cells, leading to increased production of reactive oxygen species and, as a consequence, the expression of a variety of proinflammatory gene products, including monocyte chemoattractant protein-1 (MCP-1) (Griendling et al., 1994, 1997; Chen et al., 1998; Kranzhofer et al., 1999). Additional reports have also suggested that angiotensin II may contribute to lesion formation by altering sympathetic outflow, aldosterone secretion, and prostaglandin production (Simon and Abraham, 1995).

In our investigation, we observed that syndecan-1 was abundantly expressed in infiltrating lipid laden macrophages found in atherosclerotic lesions induced upon initiating infusion of

angiotensin II in ApoE deficient mice fed an atherogenic diet. While syndecan-1 mRNA is activated in balloon-injured arteries (Nikkari et al., 1994) and syndecans released from the cell membrane appear to enhance SMC migration in vitro (Li and Chaikof, 2002), this is the first report of syndecan-1 expression in atherosclerotic lesions. Correlative in vitro studies confirmed that angiotensin II regulates the expression and shedding of syndecan-1 in macrophages in a time- and dose-dependent manner. Although studies focused on factors that regulate macrophage syndecan-1 expression have been limited, a recent report has noted that the chemokine, stromal cell-derived factor-1 (SDF-1; CXCL12), may also accelerate macrophage syndecan-1 shedding (Brule et al., 2006).

Cells regulate expression of syndecans via complex mechanisms that are not completely understood. Previous studies of syndecan-1 turnover in response to other stimuli and our current understanding of the signaling pathways that are activated in response to angiotensin II provided motivation to examine the role of MAP kinase signaling in angiotensin II-induced syndecan-1 mRNA expression. In particular, many of the proatherogenic-proinflammatory effects mediated by angiotensin II occur via MAP kinases that are either activated directly by AT1 receptor signaling or via reactive oxygen species and hydrogen peroxide produced by angiotensin II mediated activation of membrane bound NAD(P)H oxidases (Mehta and Griendling, 2007).

MAP kinases are a family of serine/threonine kinases, including ERK1/2, p38 MAP kinase, and JNK/SAPK, involved in the conduction and amplification of critical cellular signaling pathways. Our investigations demonstrated that the ERK1/2 and JNK/SAPK MAP kinase pathways, but not p38 MAPK related signaling, are involved in angiotensin II-induced expression of syndecan-1 mRNA. Angiotensin II-mediated MAPK activation is followed by an increase in c-Fos, activated by ERK, and c-Jun, activated by JNK, and, as a consequence, increased AP-1 activity is observed due to dimerization of c-Jun and c-Fos (Mehta and Griendling, 2007). Significantly, Jaakkola et al. (1997) have reported that upregulation of syndecan-1 expression may be driven by an AP-1 dependent enhancer complex located in a 5' region proximal to the syndecan-1 promoter.

We also observed that angiotensin II upregulation of syndecan-1 mRNA could be attenuated by the PI3K pharmacologic inhibitor, LY294002. PI3K has been implicated in angiotensin II induced expression of genes associated with the progression atherosclerosis (Seshiah et al., 2002; Mehta and Griendling, 2007). Indeed, consistent with PI3K-dependant activation, the proximal promoter of the syndecan-1 gene is controlled by members of Sp1-transcription factor family, which can be activated by PI3K (Lam et al., 2003; Ornskov et al., 2007).

It has been established that accelerated syndecan shedding is a regulated host response to tissue injury and shed syndecan ectodomains have recently been identified as regulators of inflammation. It is likely that shed syndecans are capable of sequestering a variety of heparin-binding pro-inflammatory chemokines, such as monocyte chemoattractant protein-1 (MCP-1) and RANTES, within the extracellular matrix. Although chemokines are usually thought to act as soluble mediators of atherogenesis, gradients of soluble unbound proteins are not stable in vivo, and it is likely that the presence of immobilized "solid phase" gradients underlies the recruitment, activation, and migration of leukocyte populations and also influences smooth muscle cell behavior. Recent investigations have also demonstrated that MMP-2, 7, 9, and 13 can all bind to heparin with dissociation constants (Kd) as low as ~5–10 nM (Yu and Woessner, 2000). Binding of MMPs to heparan sulfate in the extracellular space could prevent loss of secreted enzyme, provide a reservoir of latent enzyme, and regulate enzyme levels (Yu and Woessner, 2000). Shed syndecan ectodomains likely have important physiologic roles in normal tissue repair. Nonetheless, stimuli that accelerate shedding may lead to maladaptive responses that play a critical role in vascular lesion formation. Thus, it is possible to speculate

that the expression and shedding of syndecan-1 by infiltrating macrophages in response to locally generated angiotensin II potentiates an early inflammatory response, as well as the proteolytic environment found within atherosclerotic lesions.

In summary, macrophages expressing syndecan-1 are observed in atherosclerotic lesions in a murine model of angiotensin II induced atherosclerosis. Elevated levels of syndecan-1 may be attributable, at least in part, to angiotensin II. Given the established functional properties of this family of heparan sulfate proteoglycans, it is plausible that chronically accelerated syndecan-1 shedding by infiltrating macrophages could potentiate vascular wall lesion formation by promoting the generation of a sustained proinflammatory, proteolytic, growth-stimulating environment.

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Fig. 1.

Mac 3 and syndecan 1 immunohistochemical staining of the thoracic aorta of ApoE^{-/-} mice 8 weeks after infusion of angiotensin II. A: Nonimmune rat IgG control for Mac3. B: Mac3 staining. C: Nonimmune goat IgG control for syndecan-1. D: Syndecan-1 staining. Analysis is representative of atherosclerotic lesions in six mice. In atherosclerotic lesions, Mac3 positive staining and syndecan 1 positive staining were observed within identical regions. Syndecan-4 (E), but not syndecan-1 (F) was expressed within the aortic wall of age matched mice that were not subjected to either angiotensin infusion or a high fat diet.

Fig. 2.

Angiotensin II regulates the expression of syndecan-1 mRNA in a time- and dose-dependent manner in cultured macrophages. A: Histogram depicting a time course analysis of syndecan-1 mRNA expression levels by QRT-PCR after angiotensin II (100 nmol/L) administration, relative to baseline levels. Expression levels were normalized by 18S mRNA levels and compared with vehicle control at the same time point (n=3; * $P < 0.05$). B: Histogram depicting a dose-dependent analysis of syndecan-1 mRNA expression levels by QRT-PCR after angiotensin II (10–1,000 nmol/L) administration, relative to baseline levels. Expression levels were normalized by 18S mRNA levels and compared with vehicle control at the same time point (n=3; * $P < 0.05$).

Fig. 3.

Angiotensin II-induced up-regulation of syndecan-1 mRNA occurs via multiple signaling pathways in macrophages. Histograms of syndecan-1 mRNA levels by QRT-PCR in macrophages after angiotensin II (500 nmol/L, 6h) administration in the presence of ERK/MAPK(A), PI3K (B), and JNK(C) pathway inhibitors, U0126, LY294002, and SP600125, respectively, versus vehicle (DMSO). Results were normalized by 18S RNA expression and compared with baseline, non-stimulated levels ($n=3$; $P < 0.05$). As additional control experiments, blockade of the p38 or the PKC pathways by SB203580 or GF109203X, respectively, did not alter levels of syndecan-1 mRNA expression (data not shown).

Fig. 4. Syndecan-1 mRNA and protein expression are decoupled in RAW264.7 macrophages. **A:** Representative flow cytometry of syndecan-1 staining in macrophages stimulated by 500 nmol/L angiotensin II for 12 h versus non-stimulated control. **B:** Histogram depicts the fold-change in MFI for AgII-stimulated cells versus non-treated controls ($n=4$; $P > 0.05$). **C:** Levels of the shed syndecan-1 detected by slot blot in the cell culture supernatant of angiotensin II-stimulated versus non-treated macrophages. Data are from one experiment representative of three. Additionally, similar results were observed for cell surface (by FACS) and shed (by slot blot) syndecan-1 protein levels after 500 nmol/L angiotensin II stimulation for 10, 18, and 24 h.

Fig. 5.

Syndecan-1 protein expression can be induced by dbcAMP in a dose-dependent manner in cultured macrophages. **A:** Representative flow cytometry of syndecan-1 in macrophages stimulated by 0.1–1 mmol/L dbcAMP for 12 h versus non-stimulated control cells. **B:** Histogram depicts the MFI for syndecan-1 of 0.1–1 mmol/L dbcAMP-stimulated cells versus non-treated controls (n=3). **C:** Levels of the shed syndecan-1 detected by slot blot in the supernatant of the 0.1–1 mmol/L dbcAMP-stimulated versus non-treated control. Analysis is representative of three independent experiments.

Fig. 6. Angiotensin II and dbcAMP stimulation induce syndecan-1 expression and shedding. **A:** Histograms depicts the MFI of angiotensin II and/or dbcAMP-stimulated cells compared to non-treated cells (n=3). **B:** Levels of shed syndecan-1 detected by slot blot in the supernatant of the angiotensin II and/or cAMP-stimulated and non-treated cells. Analysis is representative of three independent experiments.