Osteopontin Is Produced by Rat Cardiac Fibroblasts and Mediates A_{II}-induced DNA Synthesis and Collagen Gel Contraction

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Abstract

Angiotensin II (A_{II}) is a critical factor in cardiac remodeling which involves hypertrophy, fibroblast proliferation, and extracellular matrix production. However, little is known about the mechanism by which A_{II} accelerates these responses. Osteopontin is an acidic phosphoprotein with RGD (arginine-glycine-aspartate) sequences that are involved in the vascular smooth muscle cell remodeling process. We identified the presence of osteopontin mRNA and protein in cultured rat cardiac fibroblasts and its prominent regulation by A_{II} (10^{-11} M). Osteopontin message levels were increased fourfold ($P < 0.01$) and protein fivefold ($P < 0.05$) at 24 h after addition of A_{II} (10^{-7} M). This response was inhibited by the AT_1 receptor blocker, losartan. Osteopontin mRNA levels were increased in hypertrophied ventricles from animals with renovascular hypertension (1.6-fold, $P < 0.05$) and aortic banding (2.9-fold, $P < 0.05$). To examine the function of osteopontin, we determined its effects on (*a*) the ability of cardiac fibroblasts to contract three-dimensional collagen gels and (*b*) cardiac fibroblast growth. A monoclonal antibody against osteopontin partially blocked A_{II}-induced three-dimensional collagen gel contraction by cardiac fibroblasts (64±4 vs. 86±5% in the presence of antibody, $P < 0.05$), while osteopontin itself promoted contraction of the gels by fibroblasts (71±5%, $P < 0.05$ compared with control). Either a monoclonal antibody against β_3 integrin which is a ligand for osteopontin or the RGD peptide blocked both A_{II} and osteopontin-induced collagen gel contraction. Thus, the osteopontin RGD sequence binds to β_3 integrins on the fibroblast to promote fibroblast binding to collagen. A_{II} induced a threefold increase in DNA synthesis of cardiac fibroblasts, which was completely blocked by antibodies against osteopontin and β_3 integrin, or by RGD peptide, but not by controls. Thus, A_{II}-induced growth of cardiac fibroblasts also requires osteopontin engagement of the β_3 integrin. Taken together, these results provide the first evidence that osteopontin is a potentially important mediator of A_{II} regulation of cardiac fibroblast behavior in the cardiac remodeling process. (*J. Clin. Invest.* 1996. 98:2218–2227.) Key words: osteopontin • cardiac hypertrophy • angiotensin II • cardiac fibroblasts • collagen gel contraction

Introduction

Osteopontin is an acidic phosphoprotein adhesion molecule with emerging importance in cardiovascular disease. Originally cloned from bone, osteopontin was identified recently in vasculature where it contributes to vascular smooth muscle cell (VSMC)[1] remodeling functions including migration, adhesion, and spreading, which are a response to endothelial cell damage (1–4). Osteopontin contains an arginine-glycine-aspartate (RGD) motif which allows the molecule to bind to $\alpha_v\beta_3$ and other integrins on the surface of cells (5, 6). An antibody to the β_3 subunit of the $\alpha_v\beta_3$ integrin reduced the extent of restenosis in animals and angioplasty patients (7, 8), suggesting that alteration of integrin receptor activation significantly impacts on VSMC responses to injury. Osteopontin gene expression is regulated by A_{II} and other growth factors in cultured rat VSMC (2). Osteopontin has not been identified previously in the heart, except in macrophages associated with inflammation and, more recently, in myocytes (9–11). However, its cell and matrix adhesive properties make it a useful candidate to participate in cardiac, as well as vascular, remodeling events.

There is substantial evidence that cardiac hypertrophy and remodeling are regulated by the renin angiotensin system (12–15). Mechanical stretch has been reported to enhance A_{II} production by cardiomyocytes which then hypertrophy in response to A_{II} by an autocrine mechanism (16). Cardiac fibroblasts also play an integral role in cardiac remodeling. Although the cardiomyocytes hypertrophy, the fibroblasts proliferate and increase their production of extracellular matrix (17, 18). These events increase the volume of fibroblasts and matrix relative to that of myocytes in the hypertrophied heart compared with normal heart, which ultimately results in ventricular dysfunction and heart failure (19). A_{II} AT_1 receptor mRNA levels and protein are prominently detectable in rat cardiac fibroblasts (20–22). We and others demonstrated that they mediate A_{II} effects to promote cardiac fibroblast proliferation, early growth response gene expression, and production of extracellular matrix proteins, particularly fibronectin which can bind to osteopontin (20–22). Regulation of extracellular matrix formation and composition may be one mechanism by which A_{II} regulates remodeling events in the heart. However, much remains unknown as to which adhesion proteins and receptors

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1. *Abbreviations used in this paper:* ACEI, angiotensin converting enzyme inhibitors; BrdU, bromodeoxyuridine; LVH; left ventricular hypertrophy; VSMC, vascular smooth muscle cell.

participate in the cardiac remodeling process and how these proteins impact on wound healing functions of the cardiac fibroblast.

The importance of the direct cellular effects of A_{II} is underscored by in vivo observations using angiotensin converting enzyme inhibitors (ACEI) in models of hypertrophy. ACEI both decrease and prevent the hypertrophic process resulting from hypertension by mechanisms apparently additive to the hemodynamic events associated with blood pressure lowering (13). Moreover, the progressive hypertrophy and dilation that occurs after myocardial infarction is attenuated by ACEI, which also decrease the incidence of severe congestive heart failure, recurrent myocardial infarction, and mortality after a coronary event in humans (14, 15). These results appear mediated by inhibition of A_{II}, since specific AT_1 receptor blockers equally decreased hypertrophy and interstitial fibrosis compared with ACEI in the rat heart after myocardial infarction (23).

We report herein that rat cardiac fibroblasts produce osteopontin and we address the hypothesis that this adhesion protein mediates A_{II}-regulated remodeling processes involving cardiac fibroblasts.

Methods

Animals. Adult Sprague-Dawley rats (Simonsen, CA), weighing 165–200 grams, were maintained in an environmentally controlled vivarium (23±1°C, 30–40% humidity) with a 12-h light/12-h dark photocycle. Rats were housed individually in polycarbonate cages on pine shaving bedding with free access to food and water at all times. The animals in our laboratory were cared for in accordance with the Guidelines of the Committee on Care and Use of Laboratory Animals. At the time of tissue harvest, animals were administered a pentobarbital overdose and decapitated. The heart and kidneys were rapidly removed and snap-frozen in liquid nitrogen. Tissues were stored at −80°C before use.

In some animals, blood pressures were measured using an indirect tail cuff method (IITC Life Science Instruments, Woodland Hills, CA) as described previously (24). Hypertension was induced in adult males using a two-kidney, one-clip preparation (24). Arterial clips were made from silver (2 mm wide × 10 mm long × 0.127 mm thick). Before surgery, the animals were treated with anesthetic (ketamine, 0.1 ml/100 grams of body weight, intraperitoneally; xylazine, 0.01 ml/100 grams of body weight, intraperitoneally) and antibiotic (penicillin, 0.15 cm^3/rat). The abdomen was shaved and an incision was made from the xiphisternum to the pelvis and the abdomen was opened along the linea alba. The left renal artery was exposed and dissected from the renal vein. The clip was applied with the open end away from the renal vein and the incision was closed. Sham-operated animals underwent the same procedures without application of the clip. Aortic banded animals and shams were obtained from Taconic (Germantown, NY). 3.0 surgical silk was placed around the aorta between the brachycephalic and left carotid arteries. An 18.5-gauge blunt needle was placed over the aorta, the silk was tied snugly around the aorta and needle, and the needle was slid out from under the knot. The hearts were examined 3–5 wk after banding.

Cell harvest and culture. Neonatal cardiac fibroblast cultures were prepared from Sprague-Dawley rats 1–3 d after birth and characterized as described previously (22). Briefly, neonatal hearts were dissected free of atria, minced, and subjected to trypsin and DNAse II digestion. The isolated cells were preplated for 30 min in DMEM/F12 and 5% FBS. During this period of time the nonmyocytes attached to the plate while the myocytes remained floating, thus separating the two populations. The attached nonmyocytes were grown in DMEM/F12 with 10% FBS until they reached confluency, at which time they were detached by trypsin treatment (0.5%) and split 1:4. All experiments were performed in the second and third passages after starvation in serum-free DMEM/F12 containing insulin (5 μg/ml), transferrin (5 μg/ml), and selenium (5 ng/ml) (ITS; Sigma Chemical Co., St. Louis, MO) for 48 h. Adult cardiac fibroblasts were harvested from sham-operated and aortic banded animals using a procedure described in detail (22). The ventricle was minced and incubated with 0.1% trypsin and collagenase (type IV, 300 μU/ml) at 37°C. Isolated cells were plated at the end of each 10-min digestion period. At the end of five digestion periods, all the isolated cells were combined and resuspended in DMEM/F-12 medium containing 5% FBS and plated. After a 1-h incubation, the unattached cells were removed, and the adult cardiac fibroblasts were handled similarly as the neonatal fibroblasts.

Isolation and analysis of RNA. Total RNA was isolated from nonmyocytes using guanidinium-isothiocyanate followed by phenol chloroform extraction (25). RNA was size-fractionated by electrophoresis through a denaturing 1% agarose gel, transferred to nitrocellulose membranes, and hybridized with cDNA probes labeled with [^{32}P]-dCTP (3,000 Ci/mmol) by random priming. The rat ANF probe was kindly provided by David Gardner (University of California, San Francisco), and the osteopontin probe was prepared as previously described (3). The hybridization signals of the specific mRNAs of interest were normalized to those of CHO-B, a constitutively expressed gene to correct for differences in loading or transfer (26). Quantitation of Northern blots was performed by densitometric analysis using the Gel Documentation System (model GDS 5000; UVP Inc., San Gabriel, CA) and the results were analyzed with the densitometric software (Gel Base; UVP Inc.). Several autoradiographic film exposures (from 12 h to 7 d) were used to ensure that the densities of the signals were linear on each film.

Treatment of fibroblasts. Cultured fibroblasts from the heart were studied at 70% confluence. 2 d before treatment, they were placed in serum-free media containing ITS. A_{II} (10^{-9}–10^{-6} M) was added in the presence of serum-free media for periods of 2–48 h. To assess whether the effect of A_{II} was mediated by the AT_1 or AT_2 receptor, A_{II} was added in the presence of losartan (10^{-7}–10^{-5} M), a specific AT_1 receptor antagonist, or PD 123177 (10^{-7}–10^{-6} M), a specific AT_2 receptor inhibitor. The effects of other growth factors on osteopontin mRNA levels included endothelin-1 (10^{-7} M; Bachem California, Torrance, CA), NE (10^{-5} M; Sigma Chemical Co.), EGF (50 ng/ml; Boehringer Mannheim, Indianapolis, IN), PDGF-BB (Sigma Chemical Co.), TGFβ (R&D Systems, Minneapolis, MN), and 5% FBS for 48 h. Each treatment was performed on four to six different preparations of cells in duplicate.

ELISA for osteopontin. A sandwich ELISA was developed to quantitate osteopontin levels in conditioned media using MPIIIB10, a mouse monoclonal antibody directed against rat osteopontin (Developmental Studies Hybridoma bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the National Institute of Child Health and Human Development) and OP199, a goat polyclonal antibody directed against rat osteopontin (3). 96-well plates (Nunc maxisorp) were coated with 1 μg MPIIIB10 in 50 μl PBS for 2 h at room temperature. Wells were washed with PBS and incubated with blocking solution (2% BSA/1.5% normal rabbit serum in PBS). After washing, conditioned media containing 40 μg of total protein, or known concentrations of purified rat osteopontin, were added to the wells and incubated for 2 h at room temperature, or overnight at 4°C. After washing, 250 ng OP199 was added in blocking solution and incubated at room temperature for 1 h. After washing with PBS, 50 μl of ABC solution (Vectastain Elite ABC kit; Vector Labs, Inc., Burlingame, CA) was added and incubated for 45 min at room temperature. After washing with PBS, color was developed using the chromogenic substrate 0-phenylenediamine. Reactions were stopped with 100 μl of 4.5 M sulfuric acid. Absorbance was measured at 490 nM, and concentra-

tions of osteopontin were determined by comparison with a standard curve generated using purified osteopontin on the same plate.

Western blot. Conditioned media samples were collected from vehicle-treated or A_{II}-treated cardiac fibroblasts at 2, 6, 24, and 48 h. Samples were electrophoresed and transferred to a nylon membrane, and Western blotting was performed using antiosteopontin antibody MPIIIB10 as described previously (9).

Immunocytochemistry. Cells were placed on culture chamber slides and grown in culture medium. After starving in serum-depleted medium for 48 h, cells were treated with A_{II}, serum, or kept in serum-free medium. After 48 h cells were washed twice in PBS, then slides were fixated in omnifix (Zymed Laboratories, Inc., South San Francisco, CA) for 10 min, washed three times in distilled water, and permeabilized in 0.1% Triton X-100 (Sigma Chemical Co.) for 10 min. After three washes, incubation was performed in PBS containing 5% BSA (Sigma Chemical Co.) for 60 min at room temperature. For histologic localization of rat osteopontin, OP199 at a titer of 1:1,000 (1 μg/ml) was used. Nonimmune serum was used as a control. For histologic localization of rat β_3 integrin, a monoclonal antibody against rat β_3 integrin (clone F11; Pharmingen, San Diego, CA) at a titer of 1:100 (5 μg/ml) was used (27). The primary antibody incubations were performed in 1% BSA containing PBS for 60 min. Biotinylated secondary antibody (Zymed Laboratories, Inc.) was applied for 30 min, followed by an incubation with streptavidin–peroxidase complex for 20 min. Peroxidase activity was detected using aminoethyl carbazole as a chromogen (liquid AEC kit; Zymed Laboratories, Inc.). Slides were then counterstained with Mayer's acid hematoxylin for 3 min.

Cardiac fibroblast DNA synthesis. Incorporation of the thymidine analogue, bromodeoxyuridine (BrdU), was measured to determine the effect of A_{II} on DNA synthesis (28). Cardiac fibroblasts were plated out at 3.0×10^4 cells on 24-well plates (Falcon primaria) in DMEM/F 12 with 10% FBS for 48 h. After serum starvation for 36 h in DMEM/ITS medium, incubation with A_{II}, 2.5% FBS, angiotensin II (10^{-6} M), losartan (10^{-4} M), RGD peptide or its control RGE peptide (10^{-4} M; Bachem California), or the monoclonal antibodies against osteopontin (MPIIIBIO) or β_3 integrin (F11) were performed for the next 20 h. Then, 15 μM BrdU (Sigma Chemical Co.) was added, and the incubation was continued for another 4 h. After several washes with PBS, cells were fixed with methanol (10 min at 4°C), followed by incubation in 1 N HCl for 2 h. Mouse anti-BrdU monoclonal antibody (dilution 1:400; Zymed Laboratories, Inc.) in PBS (with 2% BSA) was incubated overnight at 4°C, followed by AP-conjugated goat anti–mouse IgG antibody (dilution 1:1,000, A-1682; Sigma Chemical Co.) for 1 h at room temperature. A fast red substrate system (Dako Corp., Capinteria, CA) was used as a chromagen; counterstaining was performed with hematoxylin. Cell nuclei with BrdU incorporation appeared red and were counted in four to six different high power fields/well and related to total cell number/high power field.

Collagen gel contraction. Attached collagen gels were prepared according to the method of Guidry and Grinnell (29) using Vitrogen 100 collagen (Cetrix, Palo Alto, CA), which was adjusted to physiological ionic concentration and pH with 10× MEM (Gibco Laboratories, Grand Island, NY) and 0.1 M NaOH while maintained at 4°C. The final collagen concentration was 1.5 mg/ml. Cardiac fibroblasts (5×10^5 cells) were added to 0.2 ml of hydrated collagen gel. This number of cells was found to provide optimal conditions based on maximal reproducible gel contraction. The gels were then placed in a 12-mm–diameter circle which was scored on the bottom of each well in 24-well tissue culture plates (Falcon No. 3847). After 1 ml of serum-free medium without ITS was added to each well, the cardiac fibroblasts and collagen gels were polymerized by raising the temperature to 37°C and maintained for 60 min in a CO_2 incubator. The top of the collagen gels was overlaid with glass beads used as a reference point to measure the thickness of the gel. The height of the gel was measured on a Zeiss inverted microscope (Axiovert 135) equipped with a Mitutoyo Digimatic Indicator (series 543, 0.001–12.7 mm). The plane of focus was adjusted from the bottom of the well to the top of the gel as marked by the glass beads, and the distance of stage movement was recorded from the dial test indicator. The method was found to be reproducible to 0.02 mm.

Serum (2.5%) or A_{II} (10^{-6} M) was administered in serum-free media after polymerization of the cells and collagen. Their effects on gel contraction were examined at 24 and 48 h. Each treatment was performed in triplicate on three to six different preparations of cells. Losartan (Dup, 10^{-4} M), osteopontin (15 and 150 nM), osteopontin monoclonal antibody MPIIIB10 (7.2 μg/ml), β_3 integrin monoclonal antibody (F11, 25 μg/ml), nonimmune mouse IgG (25 μg/ml), RGD peptide or RGE peptide (10^{-4} M), and β_1 integrin polyclonal rabbit

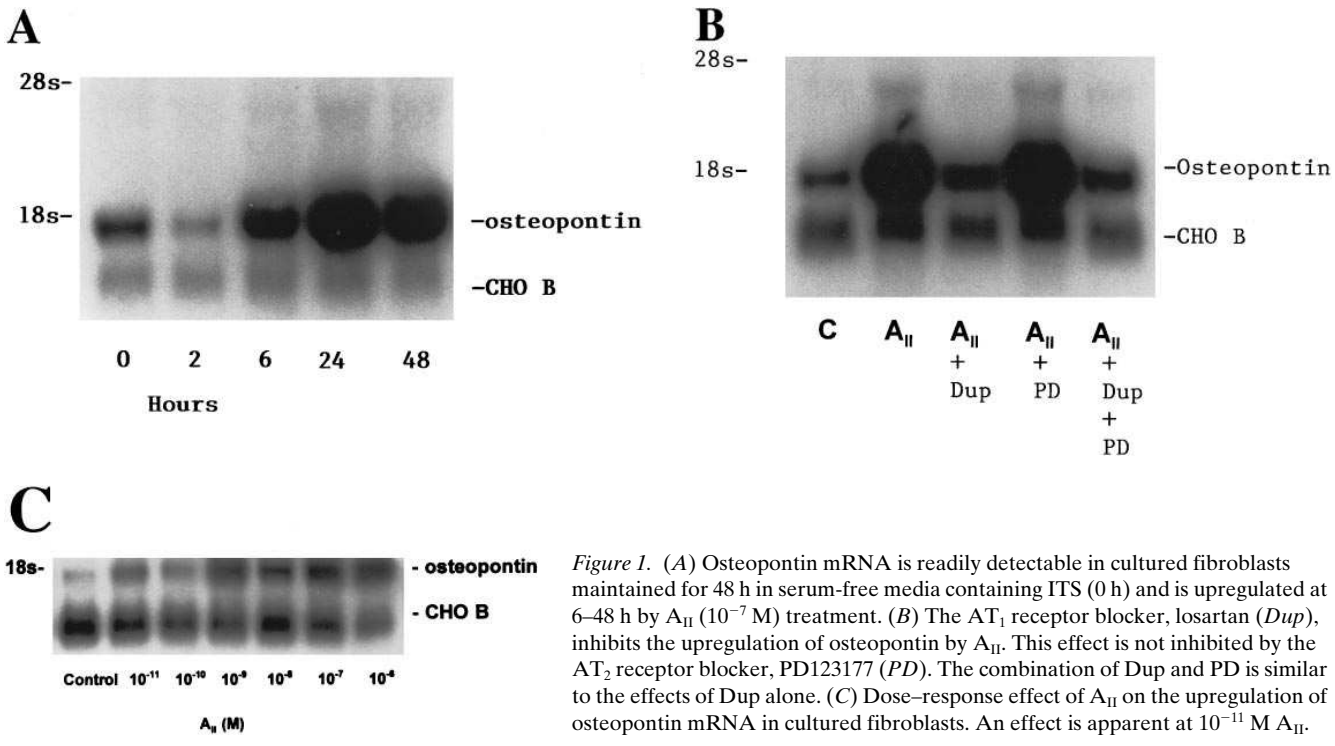

Figure 1. (*A*) Osteopontin mRNA is readily detectable in cultured fibroblasts maintained for 48 h in serum-free media containing ITS (0 h) and is upregulated at 6–48 h by A_{II} (10^{-7} M) treatment. (*B*) The AT_1 receptor blocker, losartan (*Dup*), inhibits the upregulation of osteopontin by A_{II}. This effect is not inhibited by the AT_2 receptor blocker, PD123177 (*PD*). The combination of Dup and PD is similar to the effects of Dup alone. (*C*) Dose–response effect of A_{II} on the upregulation of osteopontin mRNA in cultured fibroblasts. An effect is apparent at 10^{-11} M A_{II}.

Table I. Fold Increase in Osteopontin mRNA Levels after Growth Factor Treatment of Cardiac Fibroblast

Osteopontin	Vehicle	2 h	6–12 h	24 h	48 h
AII	0.93±0.15	0.87±0.29	3.22±0.42‡	4.47±0.83‡	3.99±0.7‡
Serum	0.80±0.11	—	3.23±0.55‡	2.72±0.50*	2.56±0.21‡
EGF	1.28±0.07	1.18±0.15	1.68±0.21	1.94±0.26	2.00±0.60
IGF-1	1.23±0.33	0.70±0.23	1.17±0.31	1.17±0.26	1.60±0.32
NE	0.86±0.09	0.70±0.20	1.70±0.64	1.56±0.37	1.88±0.48
PDGF	1.16±0.12	—	1.98±0.3	3.21±0.47*	3.15±0.39*
TGFβ	0.98±0.15	—	1.08±0.49	2.19±0.23‡	3.04±0.39*

‡$P < 0.01$; *$P < 0.05$. See Methods for explanation of treatments and statistical analysis. Densitometric readings were normalized for that of CHO-B.

antibody (20–100 μg/ml kindly provided by Thomas Borg, University of South Carolina) or nonimmune rabbit IgG (20–100 μg/ml) were preincubated for 30 min at 37°C with cardiac fibroblasts before their incorporation into the collagen gel. Pure osteopontin was prepared from neonatal smooth muscle cells as described previously (3). The height of the collagen gel after treatment was normalized against its initial height measured immediately after collagen gel polymerization. This height was compared with that of the control or vehicle sample, serum-free media, in each experiment. Results are expressed as percentage of control.

Statistical analysis. Values are expressed as mean±SEM. Group means were compared using the two-tailed Student's *t* test. Differences between treatments were analyzed statistically by ANOVA. Values with $P < 0.05$ were considered significant.

Results

Detection of osteopontin in cultured cardiac fibroblasts and regulation by A_{II}. Osteopontin mRNA was readily detectable in total RNA harvested from cultured neonatal cardiac fibroblasts (Fig. 1 *A*). A_{II} (10^{-7} M) induced a fourfold increase in osteopontin mRNA levels in cultured fibroblasts placed in serum-free media containing ITS for 48 h before treatment (Table I). Osteopontin mRNA levels increased after 6 h of treatment with A_{II}, increased further at 12 h, and remained elevated at 24–48 h. The effect of A_{II} was completely inhibited by the AT_1 receptor blocker, losartan, but was not affected by the AT_2 receptor blocker, PD123177 (Fig. 1 *B*). Similar results were seen in cultured fibroblasts from normal adult rat heart (data not shown). The effect of losartan was not altered by the presence of PD123177. The effect of A_{II} was also dose dependent (Fig. 1 *C*). Effects on osteopontin mRNA levels were seen at 10^{-11} M A_{II} and appeared to plateau at 10^{-9} M.

Immunostaining of fibroblasts grown on coverslips with antiosteopontin antibody demonstrated positive staining of all cells under quiescent conditions (Fig. 2 *B*). Staining was seen in the cytoplasm of the positive cells. No staining was seen in the fibroblasts with nonimmune sera (Fig. 2 *A*). An increase in intensity of immunostaining was seen when cells in serum-free media were treated with A_{II} (10^{-6} M) for 48 h (Fig. 2 *C*) compared with those in serum-free media alone.

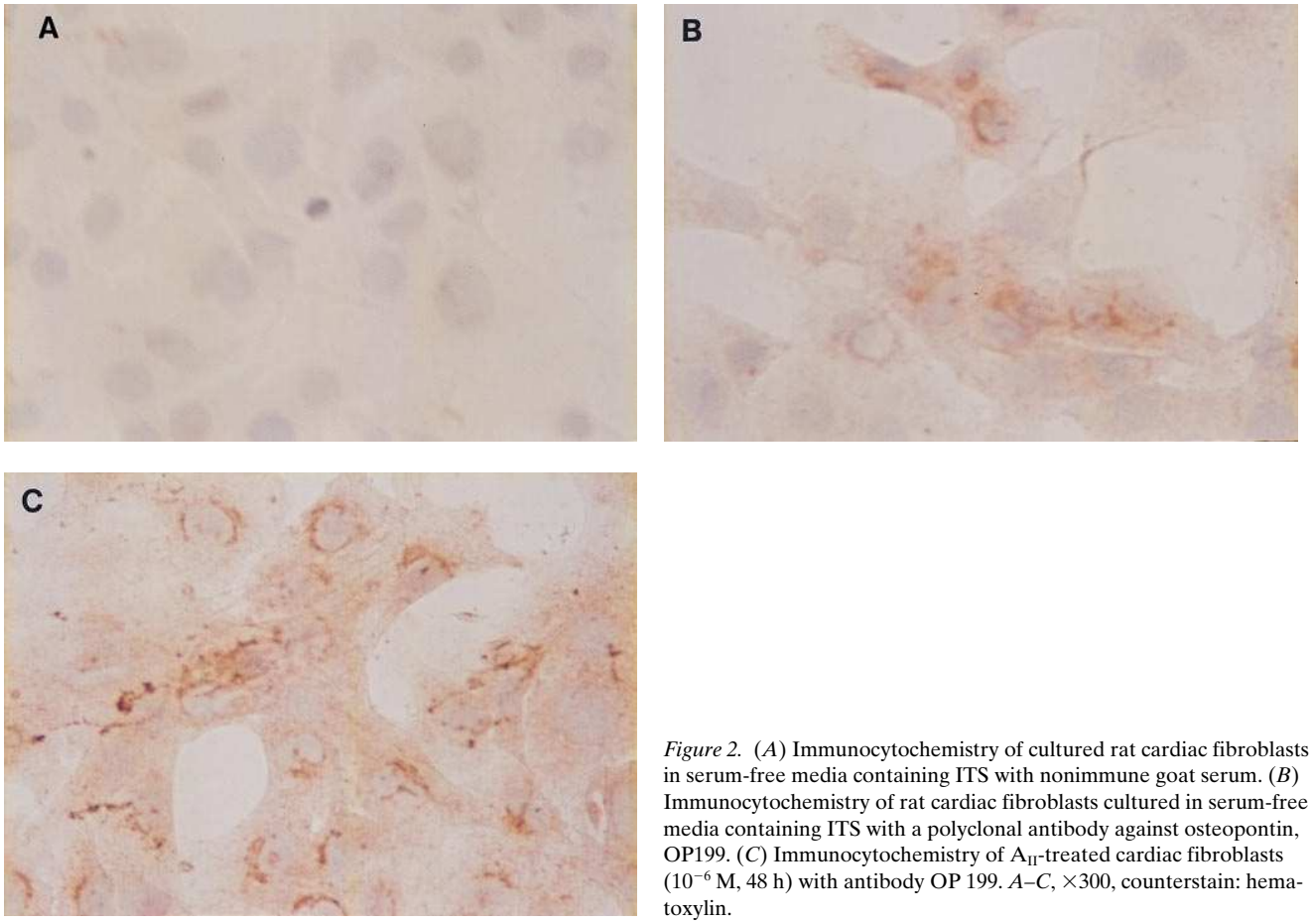

Figure 2. (*A*) Immunocytochemistry of cultured rat cardiac fibroblasts in serum-free media containing ITS with nonimmune goat serum. (*B*) Immunocytochemistry of rat cardiac fibroblasts cultured in serum-free media containing ITS with a polyclonal antibody against osteopontin, OP199. (*C*) Immunocytochemistry of A_{II}-treated cardiac fibroblasts (10^{-6} M, 48 h) with antibody OP 199. *A–C*, ×300, counterstain: hematoxylin.

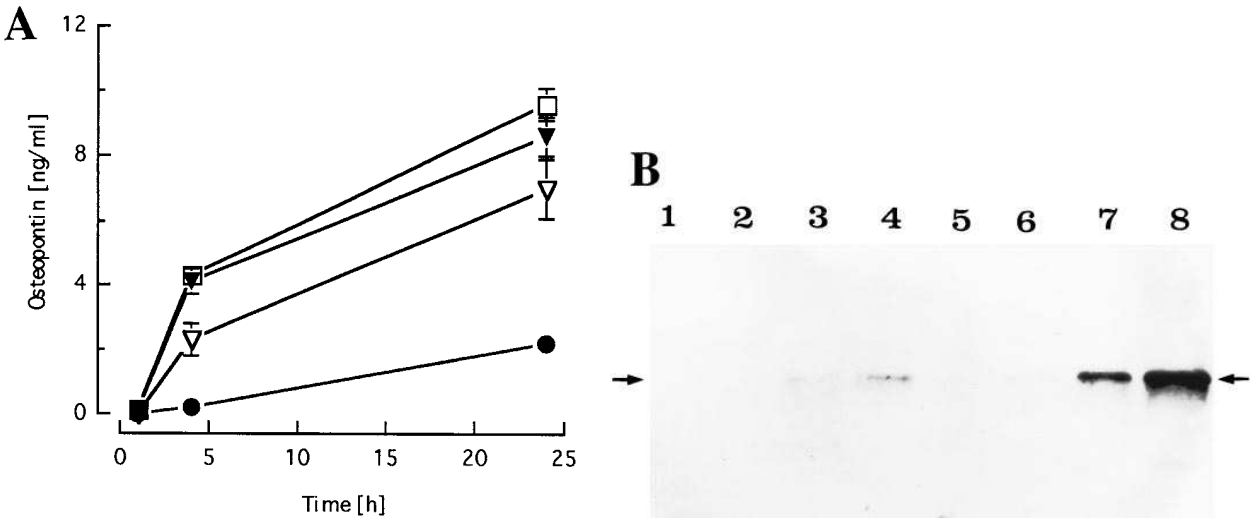

Figure 3. (*A*) Osteopontin levels (measured by ELISA) in media of fibroblasts treated with A_{II}: filled circles are serum-free media without A_{II}, open triangles are 10^{-9} M A_{II}, filled triangles are 10^{-8} M A_{II}, open boxes are 10^{-7} M A_{II}. Each point represents the average of three samples measured in triplicate. (*B*) Western blot analysis of proteins secreted by cardiac fibroblasts after A_{II} treatment. Conditioned media samples (20 μl) were collected from vehicle-treated (lanes *1–4*) or 10^{-7} M A_{II}-treated (lanes *5–8*) cardiac fibroblasts at 2 h (lanes *1* and *5*), 6 h (lanes *2* and *6*), 24 h (lanes *3* and *7*), and 48 h (lanes *4* and *8*). Samples were electrophoresed and transferred to a nylon membrane, and Western blotting was performed using antiosteopontin monoclonal antibody MPIIIB10. The arrow indicates position of migration of osteopontin at 66 kD as determined by size standards and purified osteopontin run simultaneously.

Osteopontin levels in the media of cultured fibroblasts are shown in Fig. 3. There was a progressive increase in concentrations of osteopontin in media of cells maintained in DMEM/F12 for 48 h before collection (Fig. 3 *A*). Addition of A_{II}, 10^{-9}–10^{-7} M, increased media osteopontin levels with time in a dose-dependent manner, with increasing levels seen at 24 h. This response paralleled the time course of the increase in osteopontin mRNA levels seen with addition of A_{II}. Western blot analysis confirmed the ELISA measurement of osteopontin levels in the media (Fig. 3 *B*). A 66-kD band corresponding to osteopontin was identified in the media. The intensity of the band was increased at 24 to 48 h of A_{II} treatment.

Effect of other growth factors on osteopontin mRNA levels. A_{II} was more potent at stimulating an increase in osteopontin mRNA levels than NE, IGF-1, or EGF (Table I). A_{II} increased osteopontin mRNA levels by three- to fourfold from 6 to 48 h, and 5% FBS increased osteopontin mRNA levels by two- to threefold during the same time period. These other growth factors had a tendency to increase osteopontin mRNA levels at 24–48 h; however, their effects were not statistically elevated above levels seen with vehicle alone. In contrast, PDGF-BB and TGFβ significantly increased osteopontin message levels at 24 and 48 h, but the effect of A_{II} occurred earlier and was somewhat more prominent.

Identification of β_3 integrins on cardiac fibroblasts. Immunocytochemistry staining of cultured cardiac fibroblasts grown on coverslips revealed the presence of β_3 integrins on the surface of the cells (Fig. 4 *B*) in a similar pattern as was described in rat osteoclasts (28). Staining with control IgG was negative (Fig. 4 *A*).

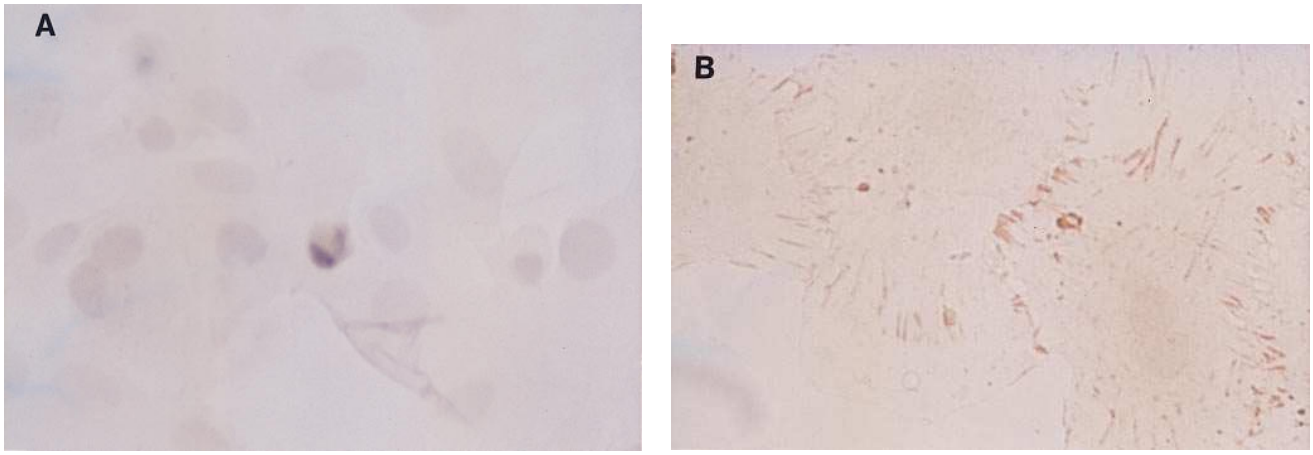

Figure 4. (*A*) Immunocytochemistry staining of cultured rat cardiac fibroblasts with control nonimmune mouse IgG (10 μg/ml). (*B*) Immunocytochemistry with a monoclonal antibody against rat β_3 integrin (F11). Note positive staining on the cell surface which appears as red lines. *A* and *B*, ×300; counterstain: hematoxylin.

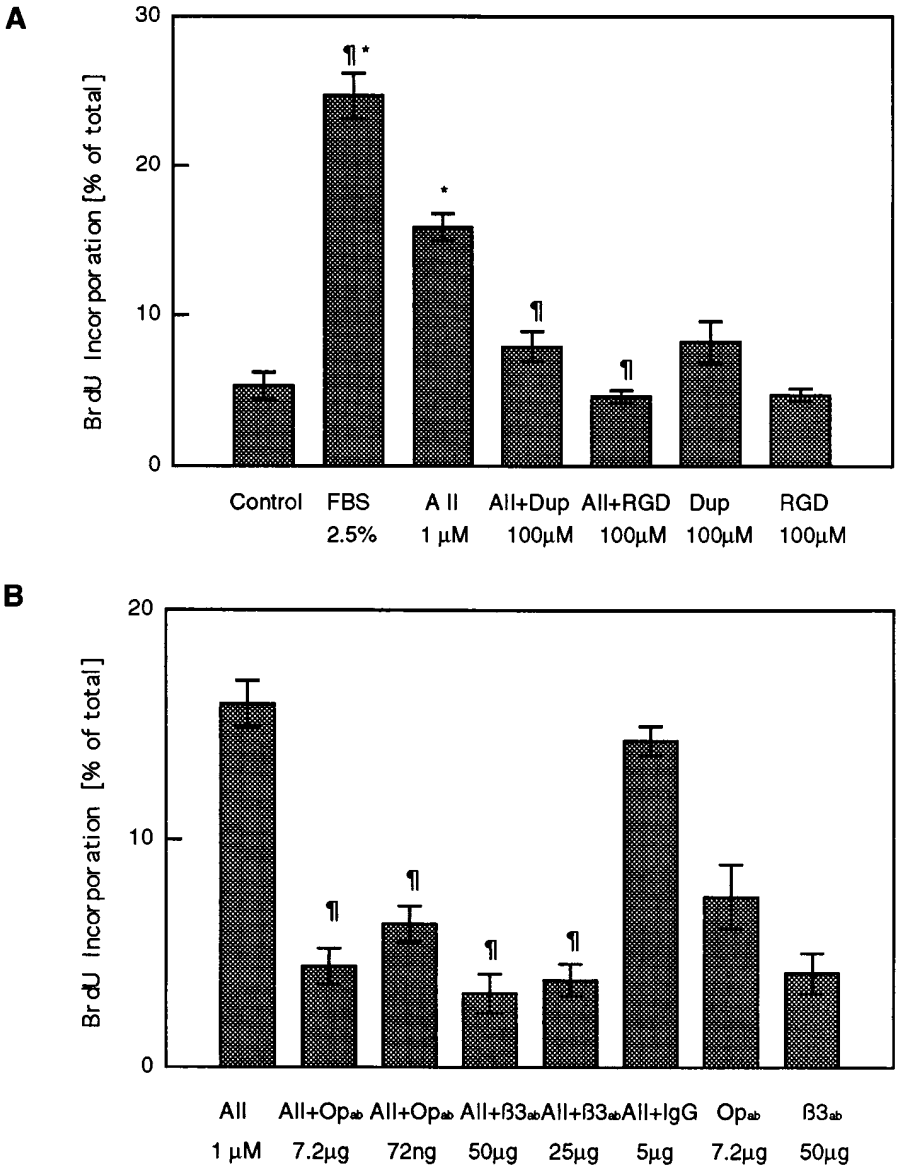

Figure 5. The effect of serum (2.5% FBS) and (A_{II}) on DNA synthesis was analyzed by incorporation of BrdU. The AT_1 receptor blocker, losartan (*Dup*), and the RGD-peptide (RGD) completely blocked A_{II}-mediated proliferation. The monoclonal antiosteopontin antibody, MP III-BIO (*Opab*), and the monoclonal anti–β_3-integrin antibody (F11 ab) also abolished A_{II}-mediated DNA synthesis, whereas control mouse IgG did not (n = 8–20, mean±SEM; *$P < 0.01$ vs. control serum-free media; ¶$P < 0.01$ vs. A_{II} alone).

Role of osteopontin and β_3 integrin in cardiac fibroblast growth. Treatment of cardiac fibroblasts for 24 h with A_{II} or 2.5% FBS significantly increased BrdU incorporation, although the effect of sera was greater than that of A_{II} (Fig. 5 *A*). The A_{II}-induced growth effect was completely attenuated by losartan (10^{-4} M) and the RGD peptide (10^{-4} M) but not affected by RGE peptide (Fig. 5 *A*). Both the osteopontin antibody (7.2 μg/ml and 72 ng/ml) and the β_3 integrin antibody (25 and 50 μg/ml), but not control IgG, also blocked A_{II}-induced DNA synthesis (Fig. 5 *B*). Neither antibody had an effect alone (Fig. 5 *B*). The effect of the antibodies and RGD peptide was not due to lack of attachment of the cells, since cell number remained the same after each treatment and BrdU labeling was only monitored in attached cells: control, 27.4±8.2 cells/HPF; A_{II}, 1 μM 24.2±10.2 cells/HPF; RGD, 24.3±5.7 cells/HPF; A_{II} + F11, 26.1±8.9 cells/HPF, A_{II} + Opab, 27.9±14.4 cells/HPF (n = 16, mean±SD).

Collagen gel contraction. This ability of fibroblasts to contract collagen gels is one indicator of their role in wound healing (28). A_{II} (10^{-6} M)-treated cells significantly contracted collagen gels at 4, 24, and 48 h when compared with vehicle alone

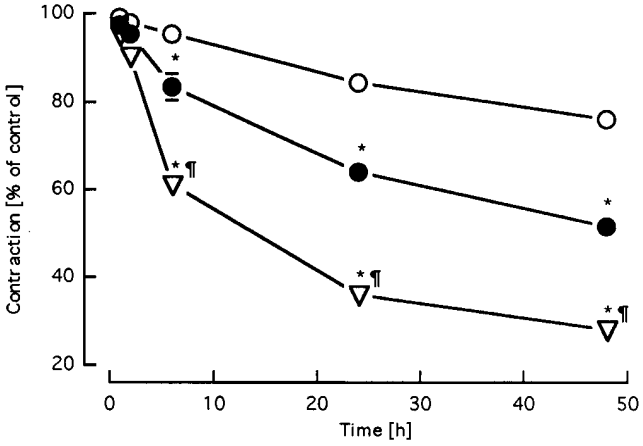

Figure 6. Collagen gel contraction by cardiac fibroblasts treated with vehicle (control, *open circles*), A_{II} (*filled circles*), or 2.5% FBS (*open triangles*). Contraction is the height of the gel at the indicated time compared with the height at 0 h and given as a percentage. Each point represents the average±SEM of four samples measured in triplicate. *$P < 0.01$ vs. control, ¶$P < 0.01$ vs. A_{II}.

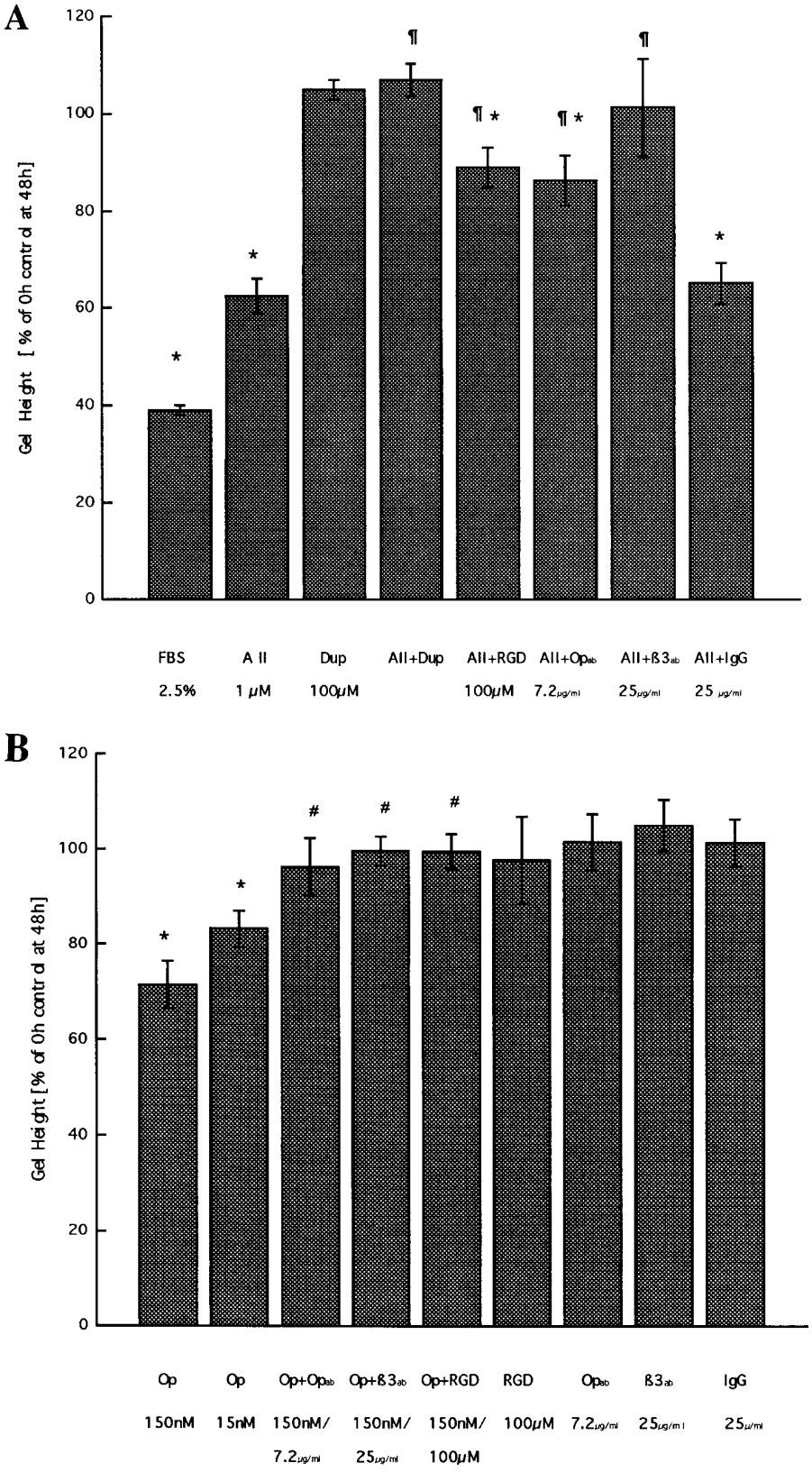

Figure 7. (*A*) A_{II} (10^{-6} M) treatment of cardiac fibroblasts enhances collagen gel contraction, expressed as percentage of control. Control represents fibroblasts treated with vehicle. Treatment with 2.5% FBS also enhances contraction. Dup had no effect alone, but completely abolished the A_{II} effect. Control IgG had no effect with or without A_{II}, while the osteopontin antibody (MPIIIBIO, *Opab*) and the RGD peptide had no effect alone, but attenuated the effect of A_{II}. (*B*) Osteopontin (*Op*) itself induced concentration-dependent contraction; this effect was completely abolished by the MPIIIBIO (*Opab*), by the F11 antibody (*β3ab*), and by RGD. A greater effect was seen with 150 nM compared with 15 nM OP. $^{*}P <$ 0.05 compared vs. control, $^{¶}P < 0.05$ vs. A_{II} alone. $^{\#}P < 0.05$ vs. OP 150 nM alone. Each bar represents the mean±SEM of four to six experiments performed in triplicate.

(Fig. 6). Cells treated with 2.5% FBS contracted collagen gels to a greater extent than A_{II} alone (Fig. 6). Based on these results, the effect of treatment was measured at 24 and 48 h, and the 48-h responses are depicted in Fig. 7. Losartan alone had no effect, but completely inhibited A_{II}-induced gel contraction (Fig. 7 *A*). In the presence of a monoclonal antibody to osteopontin, MPIIIBIO, at levels of 72 ng/ml, there was no effect on A_{II}-induced gel contraction (data not shown). However, when a higher concentration of the antibody was added (7.2 μg/ml), contraction was inhibited to 86±5% of control ($P <$

0.05 compared with A_{II} alone). Similar levels of control IgG had no effect to inhibit A_{II}-induced contraction. When added at the same doses without A_{II}, neither the osteopontin antibody nor control IgG alone had any effect (Fig. 7 *B*). RGD peptide (100 μM) also blocked A_{II}-induced contraction. Addition of osteopontin itself promoted fibroblast gel contraction; 150 nM was more potent than 15 nM osteopontin (Fig. 7 *B*). The effect of osteopontin was completely neutralized by MPIIIB10 (7.2 μg/ml) and by RGD peptide (100 μM). The monoclonal antibody (25 μg/ml) also blocked both A_{II}-induced and osteopontin-induced collagen gel contraction. The RGE peptide had no effect. A polyclonal antibody against human β_1 integrin also blocked A_{II}-induced gel contraction: 56±7% A_{II} alone versus 78±8% with antibody (20 μg/ml, $P < 0.05$) and 94±4% with antibody (100 μg/ml, $P < 0.01$). The antisera alone had no effect in the absence of A_{II}.

Upregulation of osteopontin mRNA in models of left ventricular hypertrophy (LVH). Systolic blood pressure as measured by tail cuff was 146±4 mmHg in rats 7 wk after renal artery clipping versus 101±4 mmHg ($P < 0.001$) in sham-operated animals. The heart to body weight ratio was 0.352±0.007 in the clipped versus 0.318±0.014 in the sham ($P = 0.027$). The animals with reno-vascular hypertension had an increase in both osteopontin and ANF mRNA levels in their ventricles relative to sham (Fig. 8). Densitometric analysis of the Northern blots demonstrated an osteopontin/CHO-B mRNA ratio of 1.6-fold ($P < 0.05$) and an ANF/CHO-B mRNA ratio of 3.1-fold ($P < 0.01$) in the hypertensive animals ($n = 6$) above the sham ($n = 6$). Animals with renal artery clipping were also studied at 1 and 4 wk after surgery. At 1 wk, neither ANF nor osteopontin messages were upregulated; at 4 wk there was an increase in ventricular ANF expression and an induction of osteopontin (data not shown).

Aortic banded animals also demonstrated an upregulation of ventricular ANF and osteopontin expression. The osteopontin/CHO-B mRNA ratio was 2.9 ($P < 0.05$) and the ANF/CHO-B mRNA ratio was 30 ($P < 0.01$) in the banded compared with sham-operated animals. The heart to body weight ratio was 0.453±0.138 in the banded versus 0.391±0.135 in the sham ($P < 0.01$). Cardiac fibroblasts were harvested from banded and sham-operated animals ($n = 4$ in each group) and subjected to culture. The osteopontin/CHO-B ratio was 0.88±0.12 in quiescent fibroblasts (48 h in serum-free media) from banded animals compared with 0.37±0.7 ($P < 0.05$) in the fibroblasts from shams. A_{II} (10^{-6} M) resulted in a 2.2±0.2-fold increase in osteopontin mRNA levels at 24 h and a 3.1±0.2-fold increase in osteopontin levels at 48 h compared with 0 h. Similar treatment of shams resulted in a 2.4±0.3-fold increase at 24 h (NS from banded) and a return to baseline at 48 h ($P < 0.01$ compared with banded). Thus, the A_{II} effect was more sustained in cells from the banded animals. Untreated fibroblasts from banded animals contracted collagen gels more compared to fibroblasts from sham animals (Fig. 9). To illustrate differences between fibroblasts from banded versus sham animals, gel height as a percentage of the 0 h control at 48 h is given on the *y* axis. The gel height in fibroblasts from banded hearts was half that of fibroblasts from the sham hearts ($P < 0.01$). FBS and A_{II} promoted significant contraction compared with vehicle in the fibroblasts from both banded ($P < 0.05$ for both treatments) and sham ($P < 0.05$ for both treatments) animals. Thus, gel contraction was greater under basal conditions or after FBS or A_{II} treatment in fibroblasts from banded versus sham animals.

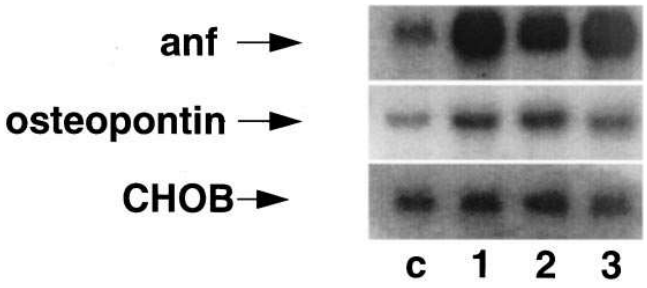

Figure 8. Representative Northern blot of mRNA levels of osteopontin, ANF, and CHO-B in ventricles of sham-operated (*left lane*) and two-kidney, one-clip renal vascular hypertensive rats at 7 wk after surgery (*three right lanes*). There is an upregulation of osteopontin and ANF in the hypertrophied hearts from the hypertensive animals.

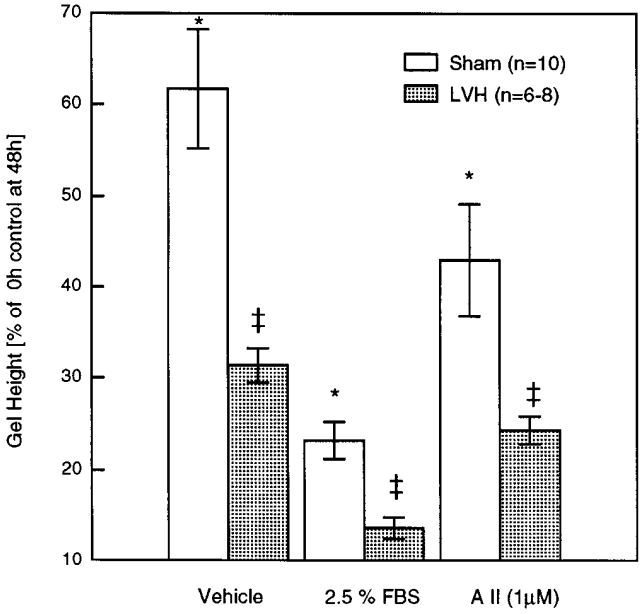

Figure 9. Measurement of gel contraction, expressed in percent change of gel height to baseline (0 h) at 48 h in fibroblasts obtained from hearts of aortic banded rats (*LVH*) and from sham animals (*Sham*). Fibroblasts from LVH demonstrated a greater extent of spontaneous contraction (vehicle), compared with fibroblasts from sham animals ($P < 0.01$). FBS 2.5% and A_{II} both induced significant contraction of the gels by fibroblasts from both banded ($^{‡}P < 0.05$ vs. vehicle) and from sham animals ($^{*}P < 0.05$) at 48 h.

Discussion

This is the first report of osteopontin expression in cultured neonatal and adult rat cardiac fibroblasts and its potent regulation by A_{II}. Moreover, we found that osteopontin mRNA was upregulated in hearts hypertrophied due to pressure overload caused by renovascular hypertension or by aortic banding. The induction of osteopontin paralleled the increase in ventricular ANF expression, suggesting that increased osteopontin mRNA levels are associated with cellular events occurring during hypertrophy. To further evaluate this possibility at a cellular level, we tested the effect of monoclonal antibodies against os-

teopontin on A_{II}-induced DNA synthesis and collagen gel contraction by cardiac fibroblasts. These antibodies completely inhibited DNA synthesis and most of the gel contraction resulting from A_{II} treatment; this observation is consistent with our finding that osteopontin itself promoted fibroblast collagen gel contraction. The AT_1 receptor blocker, losartan, completely inhibited A_{II}-induced growth and A_{II}-mediated gel contraction. In addition, we demonstrated the presence of β_3 integrins on the cell surface of cardiac fibroblasts. Antibodies against β_3 integrin and the RGD peptide also inhibited A_{II}-induced growth and both A_{II} and osteopontin-induced collagen gel contraction. We also found that fibroblasts from the hearts of banded animals contacted collagen gels to a greater extent under basal and stimulated conditions compared with fibroblasts from shams, and they tended to have a more prolonged osteopontin mRNA response to A_{II} stimulation. These results support a potentially important role of osteopontin and the β_3 integrin in cardiac remodeling, particularly in those processes regulated by A_{II}.

Substantial evidence suggests that osteopontin participates in vascular remodeling phenomena, including endothelial cell adhesion and VSMC growth, differentiation, and migration (30). Endothelial cells adhere and migrate to osteopontin (3); these actions may be relevant to endothelial cell regeneration and neovascularization (30). In addition to these vascular effects, osteopontin promotes in vitro attachment and spreading of osteoblasts, osteoclasts, fibroblasts, epithelial cells, and tumor cells (31). Antisense osteopontin RNA reduced tumorigenicity when transfected into transformed fibroblast cells (32). These observations and studies of the tissue localization of osteopontin have led to the suggestion that osteopontin participates in the repair process or in systems where there is continuous cell replacement and remodeling, e.g., gastrointestinal mucosa, bone, and uterine endometrium (33, 34).

In this investigation, A_{II} was substantially more potent than EGF, NE, or IGF-1 and slightly more potent than PDGF-BB and TGFβ in stimulating an increase in osteopontin mRNA levels in cardiac fibroblasts. Thus, although EGF and IGF-1 stimulate fibroblast proliferation (22), they do not significantly upregulate osteopontin mRNA levels. Therefore, an increase in osteopontin production does not necessarily accompany cardiac fibroblast growth as it does in VSMC, in which osteopontin is stimulated by a variety of growth factors (2). In bone cells, mechanical tension stimulates osteopontin (21); it is possible that osteopontin production by cardiac fibroblasts is related to A_{II}-induced tension on the fibroblasts. Whether PDGF-BB or TGFβ mediates some of the effects of A_{II} on osteopontin needs further investigation, since chronic infusion of A_{II} has been shown to increase cardiac TGFβ mRNA levels in vivo (35). We also found that A_{II} was more potent than EGF, NE, or endothelin at stimulating an increase in fibronectin mRNA and protein levels (22). This extracellular matrix protein is increased in models of LVH and postmyocardial infarction and is also increased in vascular injury (36, 37). Our data suggest that in the cardiac fibroblast system A_{II} regulates at least two important proteins involved in the injury response. Other studies have shown that A_{II} also enhances collagen I and III production in cultured cardiac fibroblasts (19, 20).

Further evidence suggests that osteopontin interacts with both fibronectin and collagen. Osteopontin and fibronectin can form a heat-stable complex, especially in the presence of transglutaminase, which catalyzes covalent cross-linking of these proteins (4). Fibronectin is one of the first matrix proteins laid down in the response to injury in the heart and vasculature and directs laying down of collagen fibers which bind to fibronectin (35, 38). Recent preliminary studies suggest that osteopontin forms a complex when incubated with collagen types I, II, III, and IV (4, 39). These events may be one mechanism by which osteopontin becomes stably incorporated into the extracellular matrix.

Cells bind RGD sequences on osteopontin and fibronectin which are ligands to $\alpha_v\beta_3$ and other integrin receptors on the cell membrane (40). These receptors not only mediate adhesion to matrix proteins, but engagement of these integrins activates cell signal transduction events which induce proliferation, chemotaxis, spreading, and other similar events (41). These activities involve different integrin receptors. A_{II} appears to indirectly promote these functions through its effects on enhanced production of osteopontin, fibronectin, collagen, and other matrix proteins, each of which can bind to different integrins (42). The present study provides evidence for a mechanism by which A_{II} promotes growth and collagen gel contraction of cardiac fibroblasts. A_{II} binding to the AT_1 receptor induces an increase in production of osteopontin, which then engages the $\alpha_v\beta_3$ receptor on the cardiac fibroblast surface at its RGD site. These events appear necessary for increased DNA synthesis and collagen gel contraction by the cells. Thus, osteopontin mediates at least two actions of A_{II} on the cardiac fibroblast. A previous study also implicates involvement of the β_1 integrin in collagen gel contraction because the A_{II} effect was inhibited by an antibody against β_1 integrin, and A_{II} was shown to increase β_1 integrin mobilization to the cell surface (43). The same antibody demonstrated similar results in our collagen gel contraction assay. Thus, it is likely that both β_1 and $\alpha_v\beta_3$ integrins mediate collagen gel contraction. In contrast to $\alpha_v\beta_3$, the β_1 effect is thought to be independent of the presence of RGD. Osteopontin has been identified recently in macrophages in cardiac injury models associated with inflammation in the rat (9) and hamster (10). These two reports did not identify other cell types in the heart containing osteopontin. In contrast, another report demonstrated the presence of osteopontin in cardiomyocytes and its regulation by glucocorticoid in this tissue, where it was implicated to inhibit nitric oxide production (11). In our cultured rat cardiac fibroblasts, generally all cells immunostained positively for osteopontin. We have reported previously that all cells also stained positively for fibronectin, but were negative for Factor VIII, keratin, and desmin, suggesting little contamination by endothelial, epithelial, or vascular smooth muscle cells (22). Thus, whether cardiac fibroblasts produce osteopontin in vivo needs further investigation. We found that osteopontin mRNA levels are increased 1.6- and 2.9-fold in hypertrophied hearts from renovascular hypertensive and aortic banded animals, respectively, compared with sham-operated animals. The hypertrophy was confirmed by a marked increase in expression of ANF (44). These models are not associated with inflammation, but are A_{II}-dependent. It is likely that the higher circulating A_{II} levels in these models contributed to cardiac, not macrophage, production of osteopontin or that chronic hypertrophy may be associated with cardiac osteopontin production. Histologic investigation of other models of cardiac hypertrophy will be useful to address these issues.

Whether A_{II}, osteopontin, and integrins have a similar rela-

tionship in human cardiac fibroblasts as in rat fibroblasts requires further investigation. Human atherosclerotic plaques contain osteopontin (2), and we have detected the presence of osteopontin mRNA in human heart tissue (Graf, K., and W.A. Hsueh, unpublished observations). Undoubtedly human cardiac fibroblasts contain integrins, but their type and distribution may be different in humans versus rats. Nonetheless, this investigation sheds light on potential molecular and cellular mechanisms involved in cardiac hypertrophy and remodeling and highlights a novel role of A_{II} in regulating adhesion.

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