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Research Article

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Fibroblast Growth Factor Stimulates Angiotensin Converting Enzyme Expression in Vascular Smooth Muscle Cells

Possible Mediator of the Response to Vascular Injury

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Abstract

Angiotensin converting enzyme (ACE) activity contributes to the vascular response to injury because ACE inhibition limits neointima formation in rat carotid arteries after balloon injury. To investigate the mechanisms by which ACE may contribute to vascular smooth muscle cell (VSMC) proliferation, we studied expression of ACE in vivo after injury and in vitro after growth factor stimulation. ACE activity 14 d after injury was increased 3.6-fold in the injured vessel. ACE expression, measured by immunohistochemistry, became apparent at 7 d in the neointima and at 14 d was primarily in the most luminal neointimal cells. To characterize hormones that induce ACE in vivo, cultured VSMC were exposed to steroids and growth factors. Among steroids, only glucocorticoids stimulated ACE expression with an 8.0±2.1-fold increase in activity and a 6.5-fold increase in mRNA (30 nM dexamethasone for 72 h). Among growth factors tested, only fibroblast growth factor (FGF) stimulated ACE expression (4.2±0.7-fold increase in activity and 1.6-fold increase in mRNA in response to 10 ng/ml FGF for 24 h). Dexamethasone and FGF were synergistic at the indicated concentrations inducing 50.6±12.4-fold and 32.5-fold increases in activity and mRNA expression, respectively. In addition, when porcine iliac arteries were transfected with recombinant FGF-1 (in the absence of injury), ACE expression increased in neointimal VSMC, to the same extent as injured, nontransfected arteries. The data suggest a temporal sequence for the response to injury in which FGF induces ACE, ACE generates angiotensin II, and angiotensin II stimulates VSMC growth in concert with FGF. (*J. Clin. Invest.* 1995. 95:377–387.) Key words: angiotensin II • glucocorticoids • restenosis

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1. Abbreviations used in this paper: ACE, angiotensin converting enzyme; IEL, internal elastic lamina; VSMC, vascular smooth muscle cell.

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Introduction

Angiotensin converting enzyme (ACE),[1] a zinc-containing dipeptidase, catalyzes the proteolytic cleavage of angiotensin I to angiotensin II (1). The generation of this physiologic pressor and vascular growth factor by ACE is believed to be of major importance in the maintenance of normal vascular tone. Angiotensin II also participates in angiogenesis (2), vascular remodeling (3), and the vascular response to injury (4). Expanding knowledge of the renin-angiotensin system suggests important roles for ACE in vascular diseases such as hypertension, atherosclerosis, and restenosis.

ACE activity is widely expressed in a variety of tissues including pituitary, adrenal (5), kidney, intestine (6), heart (7), and macrophages (8). In the vasculature, ACE has been thought to be expressed predominantly on the luminal surface of the endothelial cell (9). The endothelium is believed to be the major site of angiotensin II generation from angiotensin I.

The tissue renin-angiotensin system appears to be important in the vascular response to injury. Daemen (4) showed that administration of angiotensin II stimulated intimal cell proliferation. Other investigators have demonstrated that systemically administered ACE inhibitors significantly decrease intimal proliferation (10) or intimal mass (11) after balloon injury in the rat carotid and aorta. Although inhibition of circulating ACE may mediate this phenomenon, a large body of evidence suggests that the tissue renin-angiotensin system present in the vasculature may be more important (12). Vascular smooth muscle cells (VSMC) express many components of the renin-angiotensin system including angiotensinogen (13) and ACE, as shown by the binding of ramiprilate (14), and conversion of angiotensin I to angiotensin II (15). It has become clear that ACE expression is dynamically regulated in a tissue-specific manner. For example, rat cardiac ACE expression can be induced by pressure overload ventricular hypertrophy (16). In endothelial cells, high level ACE expression can be induced by density-dependent growth arrest (17, 18), hypoxia (19), glucocorticoids (20–22), endothelin (23), fibroblast growth factor (FGF) (24), increased cyclic AMP (25, 26), and ACE inhibitors (19).

To extend these findings regarding regulation of ACE to VSMC, we investigated ACE activity, mRNA and protein expression in cultured VSMC and injured vessels. ACE protein was first detected in the earliest neointimal cells of injured carotid arteries at 7 d. Using tissue culture we found that, of the multiple growth factors likely to be present during injury, only FGF stimulated ACE expression. To confirm further the importance of FGF, we transfected arteries with recombinant

FGF-1 and showed that this specifically induced ACE expression in neointimal VSMC in the absence of injury. Together these results suggest that FGF is an important regulator of ACE expression in vivo and establish a link between the renin-angiotensin system and FGF in vessel function.

Methods

Tissue culture. Rat aortic VSMC were isolated from adult Sprague-Dawley rats as described previously (27). These cells were characterized as smooth muscle based upon their growth in "hills and valleys" and expression of smooth muscle–specific α-actin mRNA (28). Cells were grown at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DME) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% (vol/vol) heat-inactivated calf serum. Dexamethasone (Sigma Chemical Co., St. Louis, MO) was prepared as a stock solution of 10 mg/ml in absolute ethanol. Before treatment with dexamethasone, all cells were growth arrested for 24 h in medium containing 0.4% serum. No significant difference in growth or ACE induction was observed when cells were grown in serum that had been depleted of all steroids by charcoal treatment (not shown). Growth factors used were PDGF BB (Austral Biologicals, San Ranon, CA), basic FGF (Synergen, Boulder, CO), and TGF-β (R&D Systems, Inc., Minneapolis, MN).

Carotid artery injury and measurements of neointima formation. The left carotid artery of male Sprague-Dawley rats (450–600 g) was injured using a 2F Fogarty catheter balloon as described by Clowes et al. (29). Before killing, each rat received 1 ml of 3% Evans blue stain in saline intravenously. After 1 h, the animals were killed, blood was removed from the left ventricle, and the carotids were perfused with phosphate-buffered saline (pH 7.2) at 100 mmHg, followed by 50 ml of phosphate-buffered saline containing Bouin's fixative (1:1, picric acid/formaldehyde). The blue areas of the left carotid and the entire right carotid were harvested, fixed in Bouin's for at least 24 h, and imbedded in paraffin. The vessels shown in Fig. 2 were fixed with phosphate (0.1 M, pH 7.4) buffered paraformaldehyde (4%). After sectioning, the tissue was stained with elastica-van Gieson or hematoxylin and eosin and morphometry was performed using a digitizing pad. To measure ACE activity, the arteries were perfused with normal saline, harvested, and a 10-mm segment was isolated. The vessels were then frozen and dry weights were measured on desiccated samples. The dry weights of the 10-mm segments varied by $< 10\%$. All ACE measurements were performed simultaneously on frozen vessels.

Transfection of FGF into vessels. Pig iliac arteries were exposed and transduced with a eukaryotic expression vector encoding a secreted form of FGF-1 (kindly supplied by T. Maciag, American Red Cross, Rockville, MD) using cationic liposomes to achieve direct gene transfer exactly as described previously (30). In brief, a secreted form of the FGF-1 gene was prepared by ligating the signal sequence of the hst/KS3 (FGF-4) gene to the open reading frame of FGF-1 in the pMEX neo eukaryotic expression vector. Expression of recombinant FGF-1 was confirmed by reverse transcriptase PCR analysis (mRNA) and by immunohistochemistry (protein). Immunoreactive protein was detected primarily in the intima including the endothelium 21 d after transfection, whereas no FGF-1 protein was detected in arteries transduced with the β-galactosidase vector or RSV-CAT (30).

ACE activity assay. Cells and tissues were harvested into 4 ml of ACE buffer (0.05 M Hepes, pH 7.5, 0.1 M NaCl, 0.05% (vol/vol) Triton X-100) and sonicated for 1 min. The homogenate was then frozen, stored for up to 2 wk, and thawed before assaying for ACE. ACE activity was assayed based on the hydrolysis of [^{3}H]benzoyl-phe-ala-pro (Ventrex Laboratories, Portland, ME) using the Ventrex protocol. Samples were assayed for total cellular protein by the method of Bradford (31) and identical protein concentrations were adjusted to yield results in the linear range of substrate utilization (3–30 μg protein per assay). Activity calculations were based on Michaelis-Menton first order kinetics. ACE activity was expressed as ramiprilate-inhibitable (1 μM ramiprilate; The Upjohn Company, Kalamazoo, MI) hydrolytic activity per microgram of protein using arbitrary enzymatic units. These units represent a percent utilization of substrate, and a value of 10.0 units/μg protein is equivalent to 0.6 pmol substrate hydrolyzed/μg protein. Because the calculation used for ACE activity is logarithmic, a value of 3.0 units/μg is equivalent to 0.3 pmol substrate hydrolyzed/μg protein. ACE activity data are presented as the mean±SE with each assay done in triplicate.

mRNA preparation and analysis. Total cellular RNA was isolated by the guanidinium isothiocyanate-cesium chloride protocol (32). 25–30 μg of total RNA was size fractionated by electrophoresis on a 1% agarose, 2% formaldehyde denaturing gel. After transfer to Nytran (33), the RNA was cross-linked to the membrane using ultraviolet irradiation (Stratalinker; Stratagene, La Jolla, CA). After 4 h of prehybridization in 50% (vol/vol) formamide, 5 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate), 5 × Denhardt's (1 × Denhardt's = 0.02% [wt/vol] each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin), 50 mM sodium phosphate (pH 6.5), and 250 μg/ml sheared salmon sperm DNA at 42°C, the Nytran membrane was hybridized in the above solution containing 10% (wt/vol) dextran sulfate and 1×10^6 cpm cDNA probe for 16 h at 42°C. For hybridization to rat aortic VSMC, the 1,033-bp EcoRI fragment of the mouse ACE cDNA clone ACE.31 (34) was used. All Northern blots were reprobed with the rat glyceraldehyde phosphate 3′ dehydrogenase cDNA (a full-length rat cDNA, clone pRGAPD 13 [35]). Appropriate cDNAs were radiolabeled using a random primer labeling kit (GIBCO BRL, Gaithersburg, MD) as per the manufacturer's protocol with [α-^{32}P]dCTP (specific activity, 3,000 Ci/mmol; Du Pont/New England Nuclear, Boston, MA) and purified using polyacrylamide chromatographic spin columns (Bio-Rad Laboratories, Richmond, CA). After hybridization, the Nytran membrane was washed three times in 2 × SSC, 0.2% SDS (15 min, 25°C) and twice in 0.1 × SSC, 0.1% SDS (15 min, 60°C). The membrane was then exposed to Kodak X-Omat AR x-ray film with an intensifying screen at −70°C for 16 h.

Immunohistochemistry and immunocytochemistry. Rabbit anti–mouse ACE polyclonal antibodies were prepared as described previously (36). Rabbit anti–human FGF polyclonal antibody was purchased from Upstate Biotechnology Inc., Lake Placid, NY). Rabbit anti–mouse α-smooth muscle actin monoclonal antibody was purchased from Sigma Chemical Company and was used as a marker for VSMC. The processing of tissue and preparation of sections was performed as described above. Immunohistochemistry was carried out in tissue sections using the same protocol previously described for tissue culture (17). In brief, after processing, sections were preincubated with rabbit nonimmune serum, covered with 1:100 dilutions of anti-FGF or anti-ACE antibody for 15 h at 4°C, washed with saline, and incubated with biotinylated anti–rabbit goat IgG (1:1,000). After washing, the sections were developed with horseradish peroxidase and H_2O_2 using the Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). To control for nonspecific staining, sections were incubated without the primary antibody. The rabbit polyclonal antibody is specific for both rodent and porcine ACE, as shown by detection on Western blots of single protein bands of the correct molecular weight (37). Quantitation of immunoreactive ACE was performed by image analysis as described in Table I. The image analysis is only semiquantitative, despite being very accurate, for several reasons. First, the relationship between the intensity of immunohistochemical reaction and ACE concentration may be nonlinear at the upper and lower limits of ACE expression. This will be determined by the antibody dilution used and conditions for detection with the Vectastain ABC kit. Second, cellular expression of ACE may vary under different growth conditions such that the relative proportions of intracellular, extracellular, and plasma membrane ACE vary, which may alter immunoreactivity. Finally, other vessel wall components (e.g., matrix present in the neointima) may alter antibody binding especially affecting staining of both ACE and background.

Data analysis and densitometry. The intensity of hybridization sig-

Table I. Image Analysis of Tissue ACE Expression

Vessel treatment	Vessel location	Relative ACE immunoreactivity
14 d after balloon	Intima-within five cell layers of lumen	928±103*
14 d after balloon	Intima-within five cell layers of IEL	358±63*
14 d after balloon	Media	112±8
Control	Media	100±11
Control	Intima	47±6‡

Tissue sections from four control and four injured vessels at 14 d after injury were used for analysis. After fixation, staining, and development as described in Methods, the sections were photographed and images were generated from color photographs. Scanning was performed with a scanner (LaCie, Inc.) at 300 dpi and image manipulation was performed using Adobe Photoshop software. Image analysis was done with gray scale images using NIH Image 1.3 software. To measure ACE immunoreactive image intensity selectively, each photograph was scanned twice, once with red light and once with blue light to generate two halftone images. The blue image preferentially detects counterstained cell nuclei and the red image preferentially detects ACE immunoreactive material. The images were converted to gray scale, noise was reduced by use of the despeckle function, and the blue image was subtracted from the red image to yield an image whose density was primarily due to the reddish brown color of the Vectastain ABC color reaction. Each vessel was divided into four quadrants and the gray scale density was obtained for randomly chosen areas within the first five cell layers of the intima nearest the lumen, within the five cell layers of the intima nearest the IEL, and within the media of both control and injured vessels. The data were normalized to the intensity of the media from the control vessels (value = 100 units). ‡ The control intima represents the value of ACE immunoreactivity in the endothelium alone, which is only a single cell layer in thickness. Thus the value relative to the other measurements should be multiplied by five. * $P < 0.05$ versus control.

nals on autoradiograms was determined by densitometry using a Silverscanner (LaCie, Inc., Beaverton OR) with Adobe Photoshop software (Adobe Systems, Inc.) and Macintosh IIcx (Apple Computer Inc.). Quantitation of autoradiogram densitometry was performed using NIH Image software (Wayne Rasband, National Institutes of Health, Bethesda, MD). Data were compared by Student's t test with a P value < 0.05 considered significant.

Results

ACE expression is increased in injured arteries. ACE expression in injured arteries was initially studied by immunohistochemistry using a polyclonal rabbit antibody raised against mouse ACE. There was a dramatic increase in ACE expression in the neointima of injured arteries at 14 d after injury (Fig. 1). It is important to note that ACE immunoreactivity showed a gradient of expression with more immunoreactivity in cells that were closest to the lumen. To quantitate this phenomenon, sections were scanned and image analysis was carried out to determine relative levels of immunoreactive ACE protein present 14 d after injury (Table I). There was a ninefold increase in ACE expression in the cells nearest the lumen and a threefold increase in cells nearest the internal elastic lamina (IEL), when normalized to the media of the uninjured vessel. The magnitude of this increase in the neointima was very significant when compared with the intima of the uninjured vessel, in which only endothelial cells were present, or to the media of the injured vessel, in which smooth muscle cells that had not proliferated were present.

In contrast to the neointima, the adventitia at 14 d showed a uniform increase in ACE expression, which was approximately equivalent to that observed in the neointimal cells farthest from the lumen. The media showed a heterogeneous increase in ACE expression. In general, there was minimal ACE expression (1.12-fold greater than control), but occasionally small clusters of cells showed very dramatic increases in ACE expression. This heterogeneous appearance of the media was a characteristic observed in all sections examined.

To determine the time course for the increase in ACE expression, vessels were harvested 6 h, 4 d, 7 d, and 14 d after injury for analysis by immunohistochemistry. As shown in Fig. 2, significant increases in ACE expression were first detected at 7 d after injury. The only cells displaying significant ACE immunoreactivity were the intimal cells present at 7 and 14 d. As in Fig. 1, the neointimal cells closest to the lumen displayed significantly greater ACE expression than did neointimal cells located closer to the IEL.

To quantify the relative increases in ACE expression observed morphologically, ACE activity was measured in injured and uninjured vessels. As shown in Table II, there was a 3.6-fold increase in total ACE activity in the injured vessel at 14 d ($P < 0.05$ vs control, $n = 4$). Because this increase was associated with a 2.9-fold increase in protein content in the injured vessel, ACE activity per microgram of protein showed a small but significant 1.3-fold increase in the injured vessel at 14 d. There is significantly more protein in the extracellular matrix of vessels 14 d after injury than in control vessels. Therefore, the increase in ACE activity per cell is likely significantly greater than 1.3-fold as suggested by the results from immunohistochemistry. This concept is supported by findings at 7 d after injury (before formation of a large neointima and accumulation of large amounts of extracellular matrix protein), when the increase in ACE activity per microgram of protein was ~ 1.8-fold. The fact that ACE activity per microgram of protein was lower at 14 d compared with 7 d appears to be explained by the preferential expression of ACE in the cell layers of the intima closest to the lumen (compare Fig. 1, *D* and *E*, with Fig. 2, *C* and *D*, see Table I also).

ACE is induced by FGF and glucocorticoids in rat aortic VSMC. To determine which of the many growth factors present in the injured vessel may contribute to the increase in ACE expression, we studied ACE activity and mRNA expression in cultured rat aortic VSMC. Of the growth factors examined, only basic FGF caused a significant increase in ACE activity (approximately fourfold at 10 ng/ml, Fig. 3). There was no significant increase in response to platelet-derived growth factor (PDGF BB), epidermal growth factor (EGF), angiotensin II, or transforming growth factor-β (TGF-β). The concentration response for induction of ACE activity by FGF showed an EC_{50} of ~ 10 ng/ml (Fig. 4), consistent with activation by high affinity FGF receptors. The induction of ACE by FGF was rapid, with onset at 12 h and peak at 48–72 h (not shown).

To investigate whether the increase in ACE activity was associated with changes in ACE mRNA, Northern blot analysis of VSMC was performed after exposure to FGF. As shown in

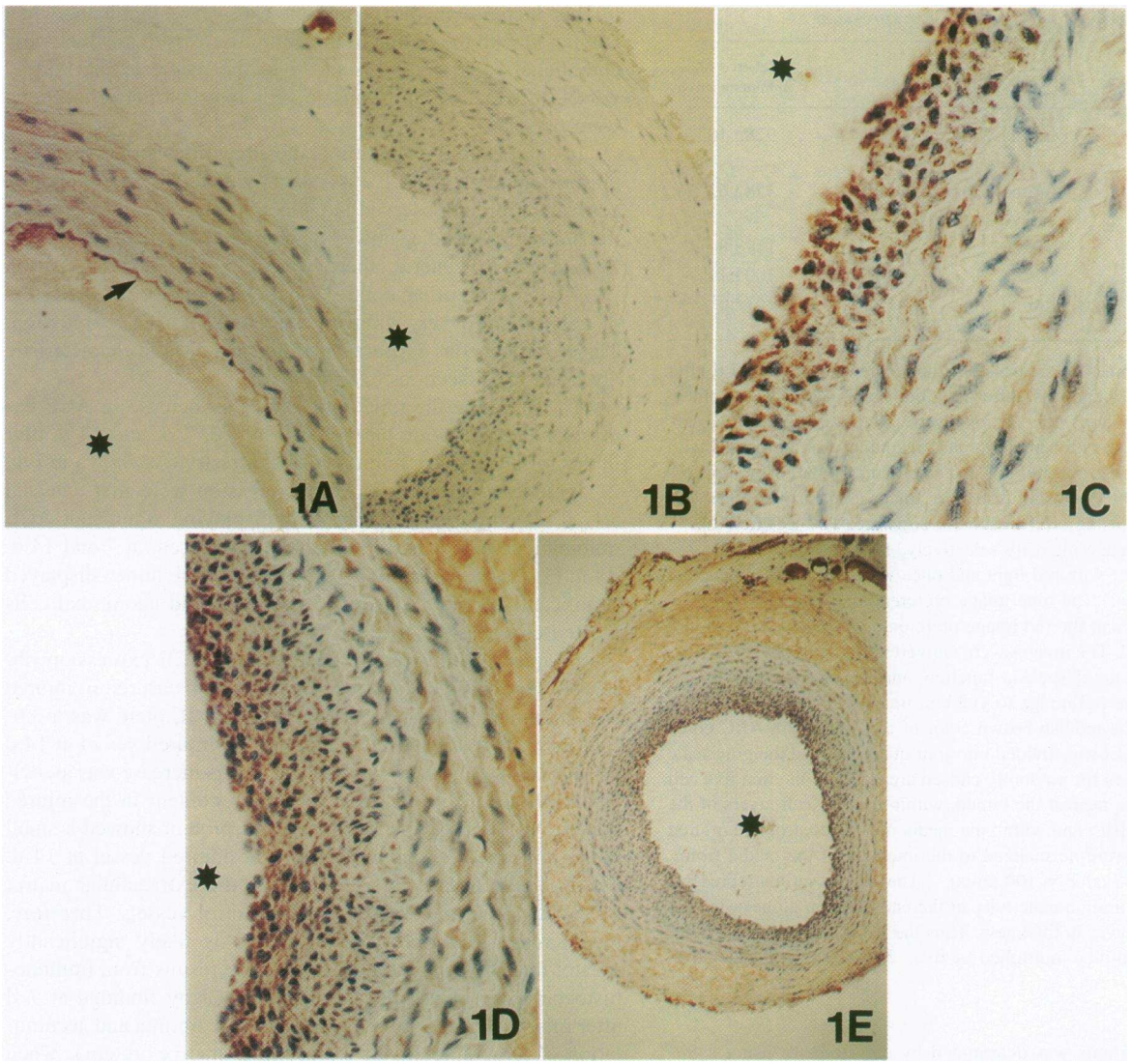

Figure 1. ACE expression in rat carotid arteries. Rat carotid arteries were harvested and prepared for immunohistochemistry with a polyclonal anti-ACE antibody as described in Methods. Vessels were fixed in Bouin's and stained with 1:100 dilution of antibody. Control uninjured vessels exhibited immunoreactivity primarily of the endothelium (*A*). No immunoreactivity was observed with preimmune serum in an injured vessel (*B*). Injured vessels exhibited immunoreactivity that was greatest in the neointima and patchy in the media (*C* and *D*). There appeared to be a gradient of ACE expression in the neointima with the greatest immunoreactivity in the most luminal cells. This is well shown in a lower power photomicrograph (*E*). In all panels the lumen is indicated by an asterisk. *A* and *B*, ×20; *C* and *D*, ×60; *E*, ×10.

Fig. 5, FGF stimulated a concentration-dependent increase in ACE mRNA expression which paralleled the increases in ACE activity.

The effect of FGF alone on ACE activity and mRNA expression is small. In previous work, we demonstrated that ACE mRNA and activity were increased by glucocorticoids (38). Because increases in stress steroids are likely part of the response to arterial injury, we studied the interaction between glucocorticoids and FGF. As shown in Figs. 5 and 6, there were increases in ACE mRNA expression and activity in VSMC when exposed to dexamethasone (30 nM) and FGF (10 ng/ml) together. Dexamethasone (30 nM) or FGF (10 ng/ml) alone stimulated 6.5-fold and 1.6-fold increases in ACE mRNA, as measured by densitometry, while together they stimulated a 32.5-fold increase ($n = 2$). Assays of ACE activity showed that alone dexamethasone (30 nM) and FGF (10 ng/ml) caused 8.0±2.1-fold (4.1±0.9 units/μg) and 4.2±0.7-fold (2.2±0.2 units/μg) increases in activity (relative to control 0.5±0.2 units/μg protein), respectively. The synergistic interaction of dexamethasone and FGF produced a 50.6±12.4-fold increase (25.3±6.1 units/μg protein, $n = 3$). We could not accurately determine a change in EC_{50} for FGF in the presence of dexamethasone as there appeared to be no maximum reached, even at 100 ng/ml, which is well above the reported value for maximal FGF effect in other responses such as FGF receptor phosphorylation (39). The synergistic interaction resulting in increased ACE expression was also apparent when steady-state mRNA levels were analyzed (Fig. 5). These findings suggest that interactions between glucocorticoids and FGF may be important to the expression of ACE after arterial injury.

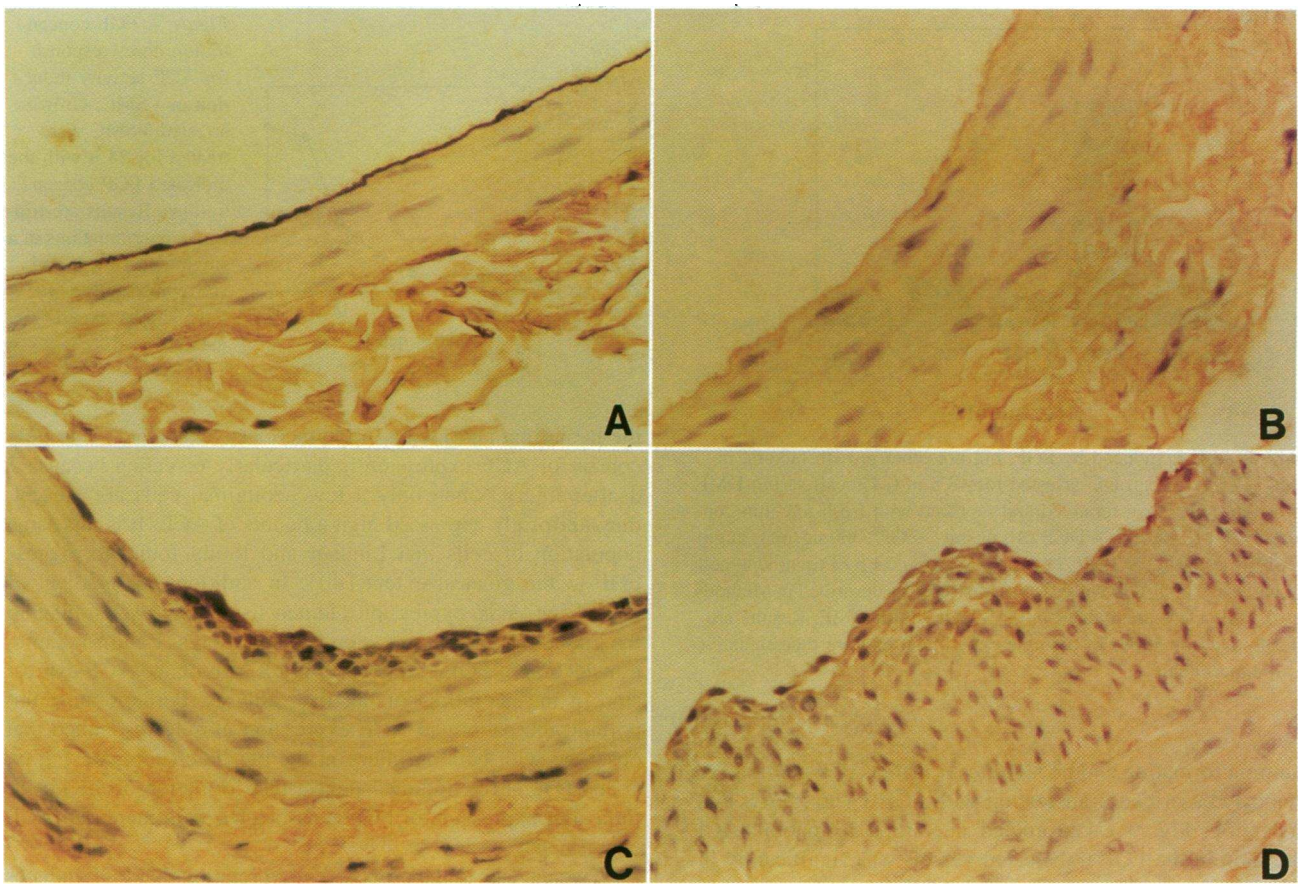

Figure 2. Time course for ACE expression in rat carotid arteries. Rat carotid arteries were harvested and prepared for immunohistochemistry with a polyclonal anti-ACE antibody as described in Methods. In contrast to Fig. 1, vessels were fixed in 4% paraformaldehyde and stained at a 1:300 antibody dilution. Control uninjured vessels exhibited immunoreactivity primarily of the endothelium (*A*). No immunoreactivity was observed with preimmune serum or in the absence of primary antibody in an injured vessel (not shown). No significant ACE activity was present in vessels harvested 6 h (not shown) or 4 d after injury (*B*). However, vessels harvested 7 d after injury exhibited immunoreactivity that was present only in the neointima (*C*). At 14 d after injury there appeared to be a gradient of ACE expression in the neointima with the greatest immunoreactivity in the most luminal cells (*D*). *A–D*, ×40.

The synergism of FGF with dexamethasone for induction of ACE activity was specific. As shown in Fig. 7 (compare with Fig. 3), only FGF acted synergistically with dexamethasone (30 nM) to increase ACE expression.

FGF and ACE expression in vivo. To analyze the interaction between FGF and ACE expression in vivo, the rat carotid artery sections from 14 d after injury were reprobed with antibodies to FGF. As shown in Fig. 8, there was minimal FGF expression in the uninjured vessel (Fig. 8 *A*). However, in the injured vessel there was increased FGF expression which was greatest in the neointima (Fig. 8, *B* and *C*). There was also a significant increase in FGF immunoreactivity in the media. FGF appeared in the media in a heterogeneous manner similar to the pattern of expression observed for ACE (compare Fig. 8 *C* and Fig. 1, *C* and *D*). Thus, there appeared to be a close spatial association between ACE expression in the injured artery and FGF expression. It is of interest that Lindner and Reidy demonstrated expression of basic FGF only in intimal cells (40), similar to the present findings for ACE (Fig. 2).

To demonstrate the ability of FGF to induce ACE expression in vivo, we performed a series of experiments in which a vector encoding a secreted form of recombinant FGF-1 was transfected into porcine iliac arteries (30). In the normal iliac artery (no injury, no transfection, Fig. 9 *A*), there was ACE protein expression only in the endothelial cells. As a control for the manipulations associated with the transfection protocol, the iliac artery was transfected with a plasmid encoding LacZ (no injury, LacZ transfection, Fig. 9 *B*). As shown (Fig. 9 *B*), there was no

Table II. ACE Activity in Injured Rat Carotid Arteries

	Protein	ACE	ACE
	μg/vessel	*units/μg*	*total units*
Uninjured	8.0±0.3	30.5±2.3	244±19
Injured, day 7	11.3±2.2*	54.6±4.2*	617±47*
Injured, day 14	23.4±5.1*	37.5±7.0*	878±119*

Vessels were harvested 2 wk after injury and ACE activity was measured as described in Methods. Results are pooled from four uninjured right carotid and two to eight injured left carotid arteries at each time point. The ACE activity for each vessel was determined in triplicate. * $P < 0.05$ versus uninjured.

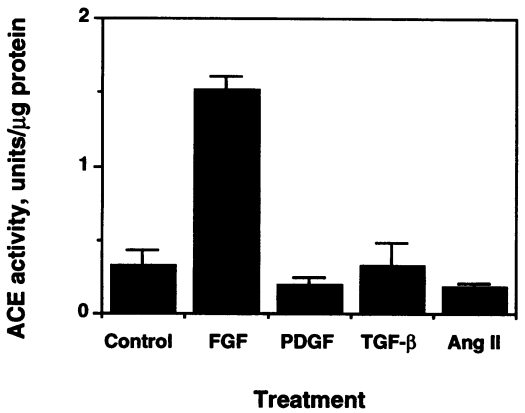

Figure 3. ACE is induced by FGF, but not other growth factors, in VSMC. Cells were growth arrested for 48 h in 0.4% calf serum/DME. The medium was then removed and replaced with fresh medium containing FGF (10 ng/ml), PDGF (10 ng/ml), EGF (300 ng/ml), angiotensin II (100 nM), or TGF-β (2 ng/ml). After 24 h of treatment, cells were harvested and ACE activity was measured as described in Methods. Control cells were maintained in 0.4% calf serum/DME. Results are triplicate determinations of a single experiment and are representative of two experiments.

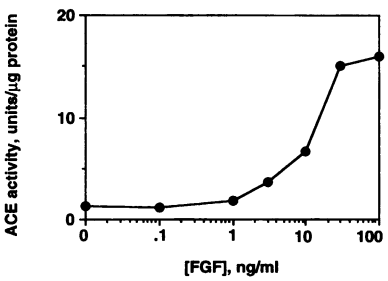

Figure 4. FGF concentration dose–response for ACE activity induction in VSMC. Growth-arrested VSMC were treated for 24 h with the indicated FGF concentrations. Results are triplicate determinations of a single experiment and are representative of three similar experiments.

neointima formation and no significant media ACE staining. As previously described, transfection with FGF-1 (no injury, FGF transfection, Fig. 9 *C*) promoted neointima formation. ACE expression in the FGF-transfected vessels was significantly increased in the neointima cells, and ACE expression appeared greatest in cells near the IEL. In response to injury (balloon dilation, no transfection, Fig. 9 *D*), there was development of a large neointima at 21 d which exhibited intense ACE expression. In contrast to the rat carotid, the greatest intensity of ACE expression was in intimal cells near the IEL, which was also observed in response to FGF-1 transfection. Thus, the only conditions to stimulate both neointima formation and ACE expression were balloon injury and FGF-1 transfection. These findings with FGF support the in vitro results for induction of ACE expression by FGF (Figs. 3–5).

Discussion

The major finding of this study is that FGF regulates ACE in VSMC. Based on tissue culture and in vivo FGF transfection experiments, we further propose that FGF is the growth factor responsible for stimulating ACE expression in the injured vessel. The increase in ACE expression was most evident in the neointima but heterogeneous increases of lesser magnitude were present in the media. The concept that the induction of ACE in the neointima is due to FGF is supported by several experiments. First, the dynamic regulation of ACE in VSMC is supported by our tissue culture experiments which show that FGF, in contrast to several other growth factors, stimulates ACE expression. The tissue culture experiments are particularly important in that serially passaged VSMC express growth factor receptors and exhibit growth responses that are reported to resemble those of neointimal VSMC (3, 4, 13, 27). Second, immunohistochemistry of the injured rat carotid shows a close spatial association between FGF and ACE expression in both the neointima and media, implying cause and effect. The time course for ACE expression is particularly revealing because 7 d after injury, when only a few neointimal cells are present, they uniformly expressed high amounts of ACE. It is this same population of cells that Lindner and Reidy found to express FGF to the greatest extent (40). In addition, the gradient of decreasing ACE expression (lumen to media) was similar to the gradient of FGF expression (compare Figs. 1 and 8). Third, transfection of FGF-1, but not of LacZ, stimulated ACE expression in the neointima of pig iliac arteries. Perhaps most important, the pattern of ACE expression closely resembled that observed with balloon injury in the pig iliac (compare Fig. 9, *C* and *D*). These findings suggest that formation of the neointima after expression of FGF is associated with increased ACE expression.

Two caveats to the proposed interpretation that FGF induces ACE expression in vivo should be made. First, the temporal correlation between FGF and ACE expression is not as close as the spatial correlation. Lindner and Reidy (40) showed basic FGF mRNA expression in the intimal VSMC as early as 4 d after balloon injury and administration of anti-FGF antibody in the immediate injury period inhibited VSMC proliferation (41). In the present study there was no increase in ACE expression at 4 d (Fig. 2 *B*). This suggests that in vivo other factors, in addition to FGF, may be required for ACE expression. Second, most of the FGF detected by the FGF antibody appears to be cytoplasmic (Fig. 8), although some matrix staining is also evident. Because FGF must bind to extracellular receptors to stimulate signal transduction events leading to cell growth and ACE expression, it is extracellular FGF that would be expected to correlate best with ACE expression. Based on these caveats, we propose that FGF and ACE are biochemical markers of proliferating VSMC in vivo and that their expression is coregulated after arterial injury.

The hormonal environment present during injury is critical to the vascular response. Increased expression of basic FGF and the FGF receptor appears to be important in rat carotid injury, since there is dynamic regulation of their expression (40, 42), and administration of an anti-FGF antibody dramatically inhibits neointima formation (41). Our data demonstrate that increased ACE expression in cultured VSMC results from the synergistic interaction between dexamethasone and FGF. Under stressful conditions, endothelial cells and vascular smooth muscle cells may be exposed to glucocorticoid concentrations which approximate those used in these experiments. For example, glucocorticoid levels (predominantly cortisol) in human plasma may increase 50-fold during surgical procedures and may be equivalent

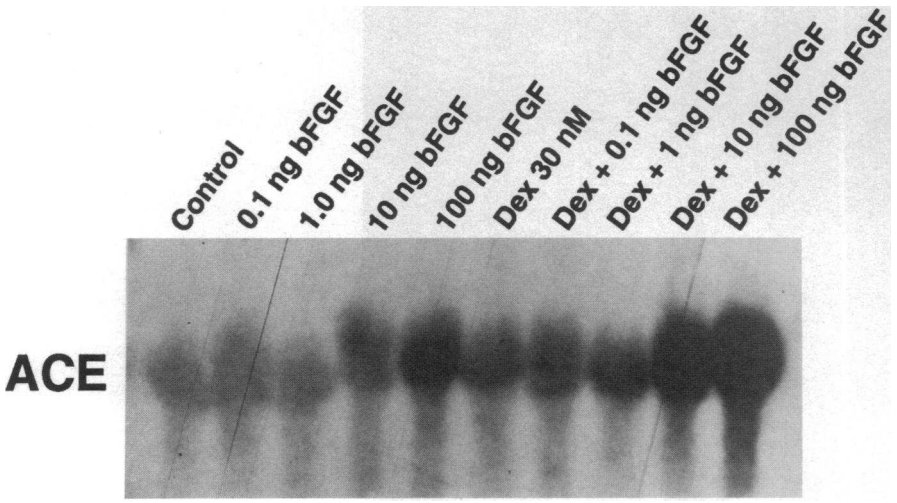

Figure 5. Concentration response for induction of ACE mRNA in VSMC by FGF and effect of dexamethasone. (Lanes *1–5*) Growth-arrested VSMC (72 h in 0.4% calf serum/DME) were treated with the indicated concentrations of FGF per milliliter of medium for 24 h. (Lanes *6–10*) During growth arrest, VSMC were exposed to dexamethasone (30 nM for 72 h), then treated with the indicated concentrations of FGF per milliliter for 24 h in the presence of 30 nM dexamethasone. Dexamethasone was dissolved in ethanol which was diluted to a final concentration of 0.1% present in all experiments. Total RNA was prepared as described in Methods and equal amounts (30 μg/lane) were then loaded and Northern blot analysis was performed with mouse ACE cDNA. Equivalent loading was verified by reprobing the blot with glyceraldehyde phosphate 3′ dehydrogenase cDNA (not shown). Results are typical of two experiments.

in glucocorticoid potency to 100 nM dexamethasone (43, 44). In cultured cells, dexamethasone alone was able to induce ACE expression to levels similar to that induced by FGF alone (compare Fig. 5 results with 100 ng FGF to 30 nM dexamethasone and Fig. 7 control results with Fig. 3 FGF results). These findings suggest that both agonists may be very important in vivo. The synergism observed in Figs. 5–7 for increases in ACE mRNA and activity suggest that dexamethasone and FGF may work by different mechanisms (e.g., protein stabilization and transcriptional regulation [38]). Further work is required to define the nature of these effects on ACE expression.

There was also significant ACE expression in some cells present in the media and adventitia. The medial cells that expressed ACE appear to be VSMC. Based on immunohistochemistry of injured vessels (Figs. 1, 8, and 9), we propose that the patchy appearance of ACE in "clusters" of VSMC may be due to local release of FGF. Possible sources of FGF may include dying or damaged VSMC and invading leukocytes, which then exert a local gradient of stimulation for ACE expression. The fact that FGF immunohistochemistry is also somewhat patchy further supports this theory. The prominent induction of ACE in the adventitia of injured vessels was unexpected. The adventitia is primarily composed of fat and fibroblast cells which normally do not express ACE (see uninjured control, Figs. 1 *A* and 9 *A*). It is of interest that these cell types appear to be capable of expressing angiotensinogen as well, based on the studies of Naftilan (13) and Weber (45). The physiologic role

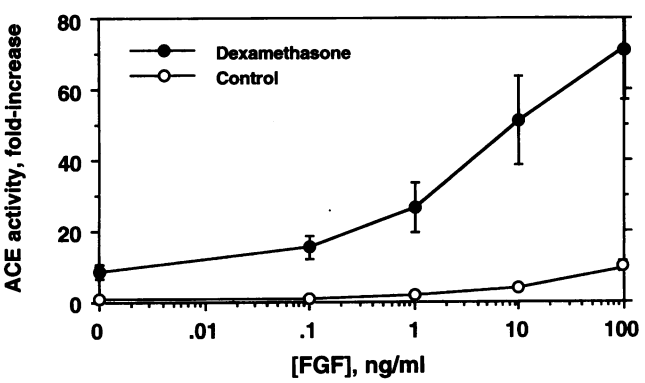

Figure 6. FGF and dexamethasone synergistically induce ACE activity in VSMC. Growth-arrested VSMC (72 h in 0.4% calf serum/DME) were treated with the indicated concentrations of FGF for 24 h (*open circles*). In addition, during growth arrest, VSMC were exposed to dexamethasone (30 nM for 72 h), then treated with the indicated concentrations of FGF for 24 h in the presence of 30 nM dexamethasone (*solid circles*). Cells were then harvested and ACE activity was measured as described in Methods. Results are triplicate determinations of a single experiment and are representative of three similar experiments.

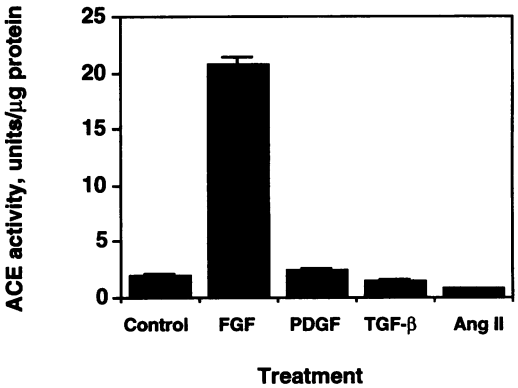

Figure 7. FGF, but not PDGF, EGF, angiotensin II, or TGF-β, acts synergistically with dexamethasone to induce ACE activity in VSMC. Cells were growth arrested for 48 h in 0.4% calf serum/DME containing 30 nM dexamethasone. The medium was then removed and replaced with fresh medium containing FGF (10 ng/ml), PDGF (10 ng/ml), EGF (300 ng/ml), angiotensin II (100 nM), or TGF-β (1 ng/ml). After 24 h of treatment, cells were harvested and ACE activity was measured as described in Methods. Control cells were maintained in 0.4% calf serum/DME containing 30 nM dexamethasone. Results are triplicate determinations of a single experiment and are representative of two experiments.

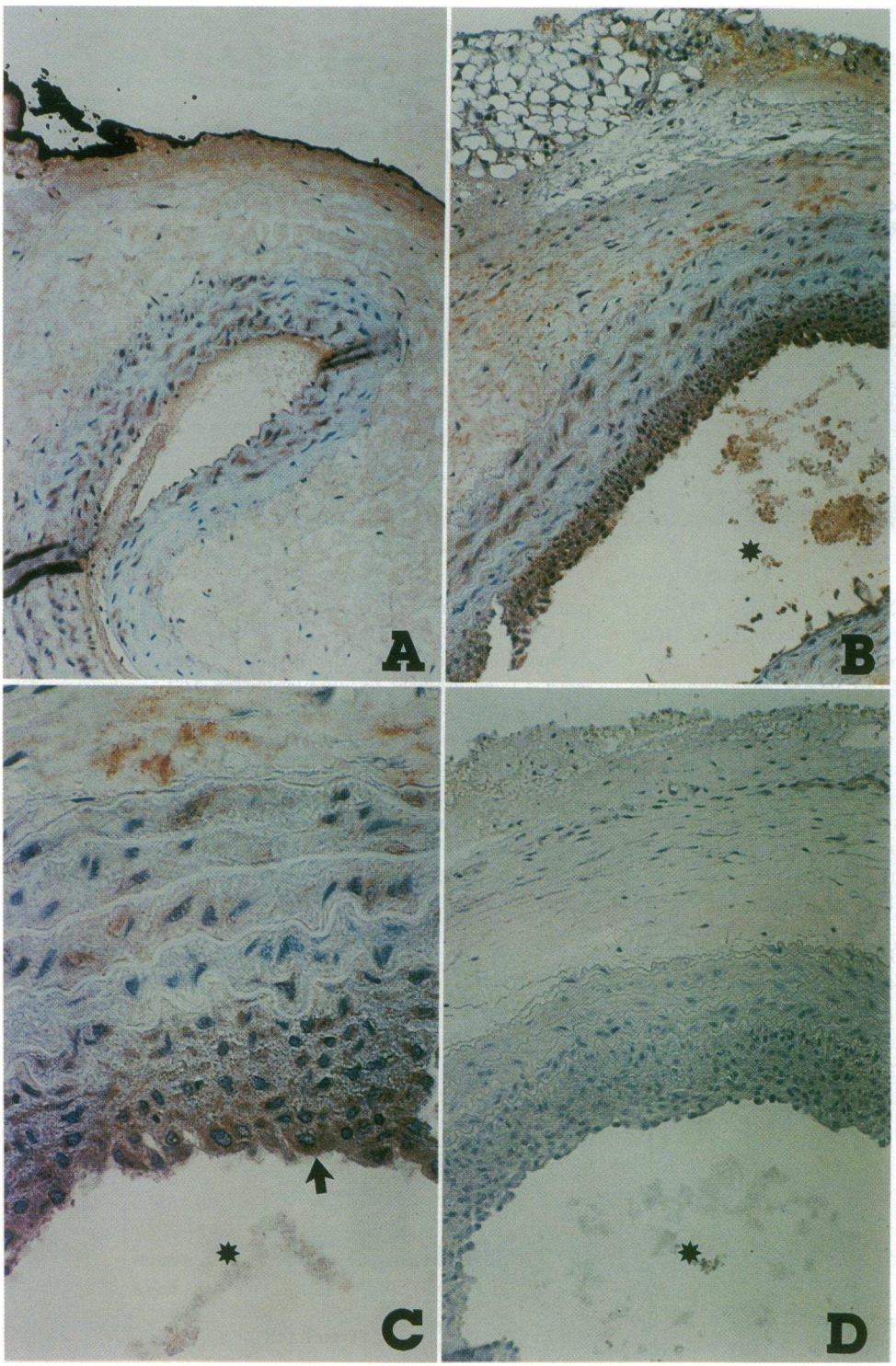

Figure 8. FGF expression in rat carotid arteries. Rat carotid arteries were harvested and prepared for immunohistochemistry with a polyclonal anti-FGF antibody (1:50 dilution) as described in Methods. Control uninjured vessels exhibited little immunoreactivity (*A*). Injured vessels exhibited immunoreactivity that was greatest in the neointima and patchy in the media (*B* and *C*). At higher power magnification (*C*) there is a gradient of FGF expression in the neointima with the greatest immunoreactivity in the most luminal cells (*arrow*). Comparison with Fig. 1 shows a similar localization of ACE and FGF in the injured vessels. Staining with no primary antibody yielded little reaction (*D*). In all panels the lumen is indicated by an asterisk. *A*, *B*, and *D*, ×20; *C*, ×60.

of the renin-angiotensin system in fibroblasts and adipocytes is currently unknown. The increase in adventitial ACE expression is likely due to induction by factors present at the site of injury. This idea is supported by the fact that highest levels of adventitial ACE expression were observed in the pig iliac arteries which had been manipulated to a greater extent than the rat carotids because of the necessity for surgical exposure.

New knowledge about the role of the renin-angiotensin system in regulation of vessel growth and remodeling supports the hypothesis that ACE regulation of angiotensin II production is important in the local response to injury. Although it is clear that angiotensin II can contribute to neointimal proliferation in the rat, the expression of the other components of the renin-angiotensin system throughout the vessel suggests a role in events other than proliferation such as inflammation, matrix dissolution, and remodeling of the vessel.

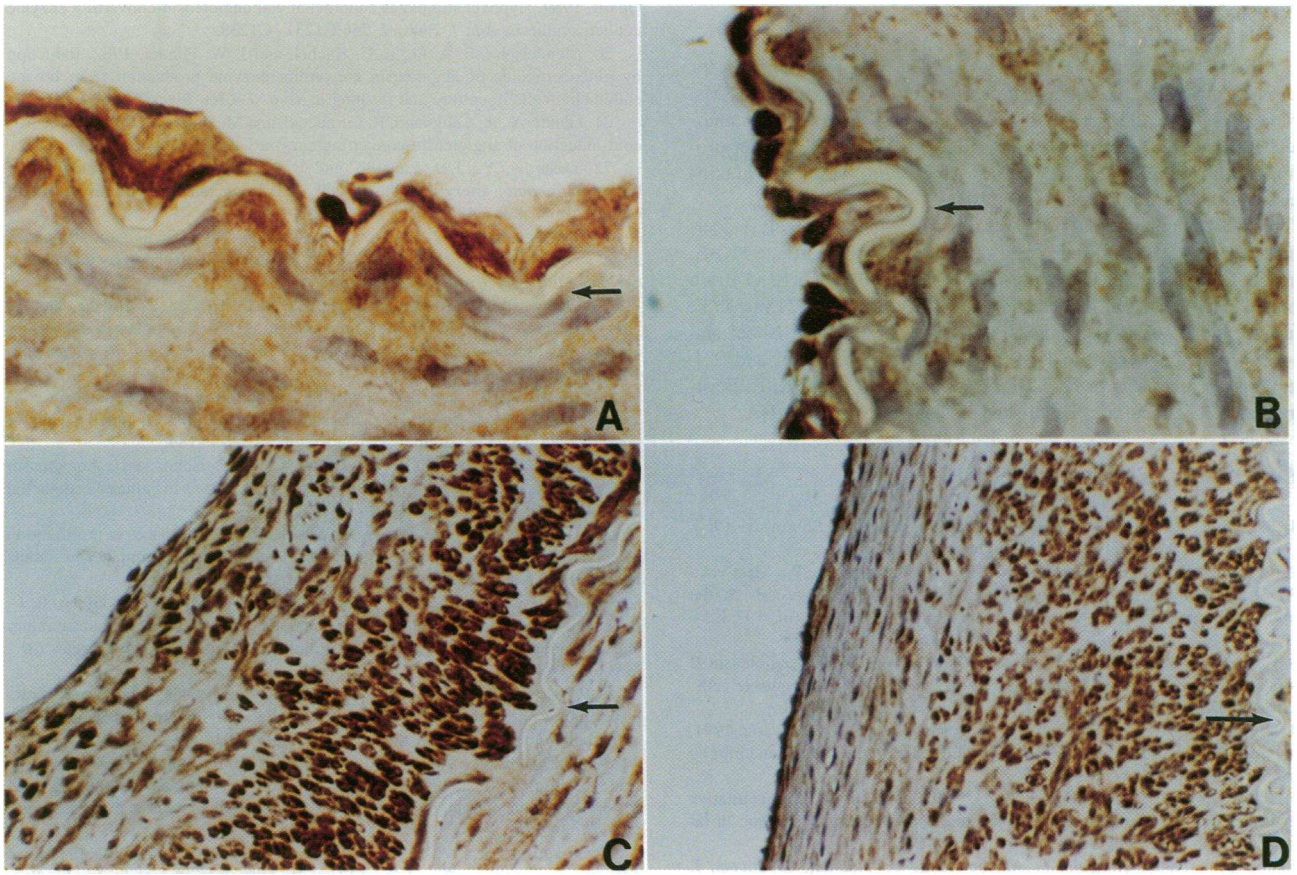

Figure 9. ACE expression in normal, injured, and transfected porcine iliofemoral arteries. Porcine iliofemoral arteries were treated as described for each panel. After 21 d, the vessels were harvested and prepared for immunohistochemistry with a polyclonal anti-ACE antibody as described in Methods. (*A*) Control vessel was uninjured and not transfected. (*B*) LacZ-transfected vessel was not injured but was transfected with LacZ as described in Methods. (*C*) FGF-1–transfected vessel was not injured, but was transfected with secreted FGF-1 as described in Methods. (*D*) Balloon-injured vessel underwent inflation with a ~ 3.0-mm latex balloon at 500 mmHg pressure for 5 min and was not transfected. In the absence of primary antibody, no staining was observed (not shown). Arrows indicate location of IEL. *A* and *B*, ×100; *C* and *D*, ×40.

Regulation of ACE specifically by FGF and not by other tyrosine kinase–coupled growth factors such as EGF and PDGF suggests unique signal transduction mechanisms for FGF. In fact, the magnitude of tyrosine phosphorylation by FGF at 5 min in cultured VSMC is considerably less than that observed with PDGF (our unpublished data). It is of interest that steroids and FGF are among the hormones thought to be most involved in development. Investigation of ACE expression during development shows regulated expression in developing vessels in the lung (46). In addition, brief treatment of hypertensive animals with ACE inhibitors at an early age affects long-term pressure (47). Thus, in many ways ACE expression as part of the response to injury is a recapitulation of development.

Our study provides insights in the interpretation of pharmacologic trials of dexamethasone, FGF antibodies, and ACE inhibitors in animal models of restenosis and human restenosis studies. For example, use of dexamethasone after angioplasty to limit restenosis showed no significant benefit (48), despite the fact that steroids inhibited neointima formation in a rabbit injury model (49). This may be due in part to the effects of dexamethasone to stimulate ACE expression as demonstrated here. This study suggests that the ability of anti-FGF antibodies to limit neointima formation in the rat carotid injury model (41) may have been due to decreased ACE expression. Finally, the failure of cilazapril to limit restenosis after angioplasty in the MERCATOR trial (50) may be explained, in part, by expression of ACE in the vessel. Although the concentrations of cilazapril used were sufficient to inhibit >90% of circulating ACE activity, they may have been inadequate to inhibit the very significant increases in tissue ACE that we demonstrated in this study. Alternatively, the effects of kinins as mediators of the beneficial effects of ACE inhibitors in rodents (51) may be different in humans.

In summary, our findings demonstrate highly regulated expression of ACE in the vessel wall. These findings agree with a recent report that demonstrated increased ACE expression after balloon injury of rat aorta (52). The stimulation of ACE expression by FGF suggests a temporal sequence for the rat carotid response to balloon injury in which FGF stimulates ACE expression which may then regulate local angiotensin II levels. The ability of angiotensin II to stimulate multiple growth factor pathways (e.g., PDGF A chain [53], insulin-like growth factor-1 [54], and TGF-β [55]) indicates that regulation of angiotensin II production is likely to be essential to the vascular response to injury.

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