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Sequential development of angiotensin receptors and angiotensin I converting enzyme during angiogenesis in the rat subcutaneous sponge granuloma

*,‡,[1]David A. Walsh, ‡De-En Hu, §John Wharton, #John D. Catravas, *David R. Blake & ‡Tai-Ping D. Fan

*Inflammation Group, Arthritis and Rheumatism Council Building, The London Hospital Medical College, Whitechapel, London, E1 2AD, ‡Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, §Department of Histochemistry, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London, W12 ONN and #Vascular Biology Center, Medical College of Georgia, Augusta, Georgia 30912–2500, U.S.A.

1 The vasoconstrictor peptide antiotensin II (AII) can stimulate angiogenesis, an important process in wound healing, tumour growth and chronic inflammation. To elucidate mechanisms underlying AII-enhanced angiogenesis, we have studied a subcutaneous sponge granuloma model in the rat by use of ^{133}Xe clearance, morphometry and quantitative *in vitro* autoradiography.

2 When injected directly into the sponge, AII (1 nmol day^{-1}) increased ^{133}Xe clearance from, and fibrovascular growth in sponge granulomas, indicating enhanced angiogenesis 6 to 12 days after implantation. This AII-enhanced angiogenesis was inhibited by daily doses (100 nmol/sponge) of the specific but subtype non-selective AII receptor antagonist (Sar^1, Ile^8)AII, and by the selective non-peptide AT_1 receptor antagonists losartan and DuP 532. In contrast, AII-enhanced neovascularization was not inhibited by the AT_2 receptor antagonist PD123319, nor was it mimicked by the AT_2 receptor agonist CGP42112A (each at 100 nmol/sponge day^{-1}).

3 AI (1 nmol/sponge day^{-1}), the angiotensin converting enzyme (ACE) inhibitors captopril (up to 100 μg/sponge day^{-1}) and lisinopril (40 μg/sponge day^{-1}), or AII receptor antagonists did not affect angiogenesis in the absence of exogenous AII.

4 [^{125}I]-(Sar^1, Ile^8)AII binding sites with characteristics of AT_1 receptors were localized to microvessels and to non-vascular cells within the sponge stroma from 4 days after implantation, and were at higher density than in skin throughout the study.

5 [^{125}I]-(Sar^1, Ile^8)AII binding sites with characteristics of AT_2 receptors were localized to non-vascular stromal cells, were of lower density and appeared later than did AT_1 sites.

6 The ACE inhibitor [^{125}I]-351A bound to sites with characteristics of ACE, 14 days after sponge implantation. [^{125}I]-351A bound less densely to sponge stroma than to skin.

7 We propose that AII can stimulate angiogenesis, acting via AT_1 receptors within the sponge granuloma. AT_1 and AT_2 receptors and ACE develop sequentially during microvascular maturation, and the role of the endogenous angiotensin system in angiogenesis will depend on the balanced local expression of its various components. Pharmacological modulation of this balance may provide novel therapeutic approaches in angiogenesis-dependent diseases.

Keywords: Angiotensin II; angiotensin receptors; angiotensin I converting enzyme; angiogenesis; inflammation; wound healing; quantitiative autoradiography

Introduction

Angiogenesis is a characteristic feature of wound repair, neoplasia and chronic inflammation. Stimulation of vessel growth facilitates wound healing and inhibition of vascularization reduces tumour growth and arthritic joint damage in some animal models (Knighton *et al*., 1986; Peacock *et al*., 1992; Fan *et al*., 1995). Angiogenesis is a multistep process, each step subject to control by a wide range of factors including regulatory peptides. Angiotensin II (AII), a vasoconstrictor peptide generated by angiotensin converting enzyme (ACE), can stimulate angiogenesis *in vivo* and endothelial proliferation and migration *in vitro* (Fernandez *et al*., 1985; Bell & Madri, 1990; Le Noble *et al*., 1993; Stoll *et al*., 1995; Hu *et al*., 1996; Munzenmaier & Greene, 1996). Understanding the role of AII in angiogenesis, therefore, may lead to novel therapeutic strategies in angiogenesis-dependent conditions.

The biological effects of AII are mediated by angiotensin (AT) receptors, of which 2 major mammalian subtypes, AT_1 and AT_2 have been identified by both pharmacological and molecular criteria (Timmermans *et al*., 1993). AT_1 and AT_2 receptors have similar affinities for AII and for the peptide antagonist (Sar^1, Ile^8)AII. AT_1 receptors display high affinity for the subtype-selective non-peptide antagonists losartan and DuP 532, whereas AT_2 receptors have high affinity for the peptide agonist CGP42112A and the non-peptide antagonist PD123319 (Whitebread *et al*., 1989; Dudley *et al*., 1990; 1991; Chiu *et al*., 1991; Brechler *et al*., 1993). Radioligand binding to AT_1 receptors is inhibited, while that to AT_2 receptors is unaffected or enhanced by dithiothreitol (DTT) (Whitebread *et al*., 1989).

AII increases vascular density in rat cremaster muscle (Munzenmaier & Greene, 1996). This response is inhibited by the selective AT_1 receptor antagonist, losartan, and enhanced by the AT_2 receptor antagonist, PD123319. AII-stimulation of endothelial proliferation is also inhibited by AT_1 receptor antagonists and by the AT_2 receptor agonist, CGP42112A (Stoll *et al*., 1995). These findings suggest that AII may either stimulate or inhibit angiogenesis through AT_1 or AT_2 receptors, respectively. Since AII has similar affinity for both AT_1 and AT_2 receptors, its net effect on angiogenesis *in vivo* will depend

[1]Author for correspondence at: Inflammation Group, Arthritis and Rheumatism Council Building, The London Hospital Medical College, Whitechapel, London E1 2AD.

on the balance of AII receptor subtypes expressed by the target tissue. AII-enhanced ^{133}Xe clearance from rat subcutaneous sponge granulomas was inhibited by the AT_1 receptor antagonist, losartan, but was unaffected by the AT_2 receptor antagonist, PD123319, suggesting that, in this tissue, AT_1 receptor-mediated angiogenesis may predominate over AT_2 receptor-mediated inhibition of neovascularization (Hu *et al*., 1996).

The development of vasoactive regulatory systems during angiogenesis remains incompletely defined. The neovasculature responds differently to vasoactive agents compared with mature blood vessels (Andrade *et al*., 1992), and receptors for the sensory neuropeptide substance P appear on maturing microvessels before innervation provides an endogenous source of the peptide (Walsh *et al*., 1996). Maturation of microvascular AII systems may also be sequential, since ACE expression is low in immature endothelial cells, and is upregulated during maturation *in vitro* (Shai *et al*., 1992). Vasoregulation by endogenous angiotensin systems will depend on the balanced local expression of its various components.

The subcutaneous sponge implant model in the rat provides a useful tool for investigating the development of peptide regulatory systems and their effects on angiogenesis (Andrade *et al*., 1987; Hu *et al*., 1995). Pharmacological agents are administered directly into the implanted polyether sponge disc via an indwelling cannula and angiogenesis is quantified sequentially as an increase in ^{133}Xe clearance from the sponge, and by morphometry following death. As the sponge is initially avascular, structures located within the boundaries of the sponge matrix must be newly-formed. Sponges are progressively infiltrated from their margins by fibrovascular granulation tissue containing blood vessels, macrophages, giant cells and myofibroblasts, and sparsely distributed lymphocytes (Walsh *et al*., 1996). Similar histological changes are observed during the healing of sutured skin wounds and in some other chronically inflamed tissues, such as synovia from patients with chronic synovitis.

In this study we have investigated the development and influence of ACE and AII receptor subtypes during neovascularization of sponge granulomas in the rat.

Methods

The sponge implant model

Circular sponge discs (1.2 cm diameter) were prepared from a sheet of 5 mm thick polyether foam. A 1.3 cm segment of polythene tubing (1.4 mm internal diameter) was secured to the interior of each sponge disc by means of 5/0 silk sutures. Before implantation, sponge discs were soaked in 70% ethanol for 2 to 3 h then transferred to sterile phosphate buffered saline (PBS). Excess PBS was removed by squeezing in a 20 ml syringe and sponges were sterilised by overnight ultraviolet irradiation.

Neuroleptanalgesia was induced in male Wistar rats (180 to 200 g) by intramuscular Hypnorm (0.5 ml kg^{-1}; 0.315 mg ml^{-1} fentanyl citrate and 10 mg ml^{-1} fluanisone, Janssen Pharmaceuticals, U.K.). On day 0, two sterile sponge discs were subcutaneously implanted in the dorsum of each rat by use of an aseptic technique as previously described (Andrade *et al*., 1987). In brief, each sponge was introduced into a subcutaneous air pouch through a 1 cm midline dorsal incision approximately 4 cm caudal to the occipital ridge. The sponge cannulae were exteriorized through needle punctures in the skin and secured in place by a 5/0 silk suture, one sponge 2.5 cm rostral to and one 2.5 cm caudal to the skin incision. The skin incision was sutured and each cannula plugged with a sterile polythene stopper. Rats were housed individually and allowed access to a normal diet and water *ad libitum*.

Test substances in 50 μl sterile PBS were administered daily through the attached cannula into each sponge from days 1 to 13 after implantation. To exclude possible acute vasomotor effects, test substances were injected into sponges at least 16 h before ^{133}Xe clearance measurements. AII was injected at 1 nmol per sponge day^{-1}, within one order of magnitude of the threshold dose for vasoconstriction in implanted sponges (Andrade *et al*., 1992). On the basis of previous *in vivo* studies and *in vitro* affinities relative to AII, AII receptor antagonists and the AT_2 receptor agonist, CGP42112A, were administered at 100 nmol per sponge day^{-1}, 2 orders of magnitude higher than the dose of AII (Whitebread *et al*., 1989; Timmermans *et al*., 1993). ACE inhibitors were administered at doses sufficient to produce tissue concentrations in excess of the therapeutic range in man (Duchin *et al*., 1988).

^{133}Xe clearance

Blood flow through sponge implants was assessed by a ^{133}Xe clearance technique, as described by Andrade *et al*., (1987) and validated by Hu *et al*., (1995). ^{133}Xe was diluted in sterile PBS to 50 μCi ml^{-1} and 10 μl was injected into the sponge implant through the cannula. The washout of radioactivity from the implant was measured for 6 min with a sodium iodide crystal positioned 1 cm above the sponge and a collimated γ-scintillation detector. Counts were recorded every 40 s by an SR8 scaler ratemeter (Nuclear Enterprises Ltd., Reading, U.K.). Percentage clearance of ^{133}Xe after 6 min was calculated as $1 - (\text{count at 6 min/initial count}) \times 100$.

Tissue preparation

Rats were killed by asphyxiation in carbon dioxide, cannulae removed by traction, the dorsum shaved and sponges rapidly excised together with overlying skin and underlying connective tissue and bisected sagitally. For analysis of fibrovascular growth areas, samples were fixed in 10% formal saline at 4°C for 1 h and embedded in paraffin wax. Four 10 μm sagittal sections evenly distributed throughout each sponge were mounted on glass slides and stained with haematoxylin and eosin.

For quantitative *in vitro* receptor autoradiography and immunohistochemistry, fresh bisected sponges and surrounding host tissues were immediately embedded in Tissue-Tek (Miles Inc., Elkhart, Indiana, U.S.A.) and frozen onto cork mounts in melting isopentane without prior fixation. Tissues were stored at −70°C until use. Sections (10 μm thick) were cut in a cryostat and thaw mounted onto Vectabond (Vector Laboratories, Peterborough, U.K.) treated slides, air dried and used immediately, or stored at −20°C with silica gel for up to 5 days until use.

Fibrovascular growth area

Fibrovascular growth areas were calculated by the axial strip sampling technique described by Mayhew and Sharma (1984) with a 4 × objective lens and a Symphony image analysis system (Seescan, Cambridge, U.K.). Images were overlaid with a 96-point square lattice with a field area of 0.66 mm^2 and a point counting procedure employed to calculate the volume fraction occupied by fibrovascular growth. Previous studies have demonstrated that changes in ^{133}Xe clearance correlate with changes in fibrovascular volume fraction, and that these indices correlate well with other indicators of angiogenesis, including vascular length density, sponge haemoglobin content and carmine dye measurements (Hu *et al*., 1995).

[^{125}I]-(Sar^{1,} Ile^{8})angiotensin II binding

Conditions for [^{125}I]-(Sar^1, Ile^8)AII binding were as previously described (Walsh *et al*., 1994). Sections of sponge implants were preincubated for 10 min in 10 mM PBS, pH 7.4, containing 5 mM $MgCl_2$, 5 mM ethylenediamine tetraacetic acid (EDTA) and 0.004% bacitracin (buffer A). Excess buffer was removed and sections were incubated for 90 min with ligand in buffer B (buffer A plus 1% bovine serum albumin). Ligand comprised 0.25 nM [^{125}I]-(Sar^1 Ile^8)AII alone (total binding), or

together with an excess (1 μM) of unlabelled (Sar^1, Ile^8)AII (non-specific binding). Following incubations, sections were washed twice for 5 min at 4°C in buffer A and rinsed in distilled water before being rapidly dried under a stream of cold air. Except where stated, incubations were performed at 22°C.

[^{125}I]-351A binding

[^{125}I]-351A was prepared as previously described by radioiodination of the tyrosyl group of the ACE inhibitor 351A (Fyhrquist *et al*., 1984). [^{125}I]-351A was purified to a specific activity of 2000 Ci mmol^{-1} by high performance liquid chromatography with a Waters C_{18} μBondapak analytical column utilizing a mobile phase consisting of 88% 0.04 M phosphoric acid with triethylamine, pH 3 and 12% acetonitrile for 10 min, followed by a linear gradient from 12% to 25% acetonitrile over the next 40 min. The retention time of the monoiodinated product was 24 min. [^{125}I]-351A was stored in the dark at 4°C for up to 2 months until use.

Conditions for [^{125}I]-351A binding were as for [^{125}I]-(Sar^1, Ile^8)AII, except that buffer A comprised 10 mM PBS, pH 7.4, buffer B was buffer A plus 0.2% bovine serum albumin, sections were incubated for 3 h at 22°C with 0.03 nM [^{125}I]-351A and non-specific binding was defined in the presence of 1 mM EDTA (Mendelsohn, 1984).

Quantification of radioligand binding

Labelled sections were apposed to radiosensitive film (Hyperfilm-^{3}H, Amersham, U.K.) and exposed at 4°C for 3 to 4 days ([^{125}I]-(Sar^1, Ile^8)AII and [^{125}I]-351A binding to skin and sponge capsule) or 12 to 14 days ([^{125}I]-(Sar^1, Ile^8)AII in the presence of DTT and [^{125}I]-351A binding to sponge stroma). Films were developed in Kodak D19 for 3 min at 15°C and autoradiographic images were analysed by a Symphony image analysis system (Seescan, Cambridge, U.K.). Tissue structures were identified by comparison of films with sections counterstained with haematoxylin and eosin, isolated by interactive thresholding then delineated interactively. Sponge stroma was defined as cellular tissue within the boundary of sponge matrix and arbitrarily classified as central stroma within the central one third of the sponge diameter, surrounded by outer stroma. Binding density was derived from grey values by comparison with 10 μm thick ^{125}I standards (Amersham, U.K.) co-exposed with each film and corrected for the activity date of the ligand.

Characterization of binding sites

Specific binding was defined as total minus non-specific binding. Characterization of specific binding was performed on PBS-treated sponges removed 14 days after implantation. Maximal binding densities (B_{max}) and K_i values for [^{125}I]-(Sar^1, Ile^8)AII binding were determined by inhibition studies, with 0.25 nM radiolabelled ligand together with 0 to 4 nM unlabelled (Sar^1, Ile^8)AII (DeBlaisi *et al*., 1989); similar affinities for [^{125}I]-(Sar^1, Ile^8)AII and (Sar^1, Ile^8)AII were assumed (Walsh *et al*., 1994).

Sensitivity of specific [^{125}I]-(Sar^1, Ile ^{8})AII binding to DTT was assessed by incubation with 0.25 nM radiolabelled ligand alone or with 10 μM losartan (AT_2 receptors remain unblocked) together with 10 μM to 30 mM DTT. Regions of stroma quantified in the absence of losartan were those to which specific binding was abolished by 10 μM losartan. Specificities of total [^{125}I]-(Sar^1, Ile^8)AII binding and of binding resistant to 10 mM DTT were further measured by inhibition of 0.25 nM [^{125}I]-(Sar^1, Ile^8)AII binding with 100 pM to 10 μM unlabelled ligands.

Microscopic localization of binding sites and immunohistochemistry

Tissue sections freshly labelled with [^{125}I]-(Sar^1, Ile^8)AII were air dried then fixed in dry paraformaldehyde vapour for 30 min at 90°C, dipped in radiosensitive emulsion ((Ilford K5) at 42°C and rapidly dried under a stream of cold air. Following exposure for 3 weeks at 4°C, emulsion-dipped slides were developed as for film autoradiograms, then counterstained with haematoxylin and eosin, dehydrated and mounted in dibutylpthalate polystyrene xylene (Raymond Lamb, London, U.K.).

For microscopic localization of [^{125}I]-351A binding, autoradiographic films were matched to consecutive haematoxylin and eosin stained tissue sections.

To investigate further the cellular localization of specific [^{125}I]-(Sar^1, Ile^8)AII binding, vascular endothelium was localized in consecutive sections to those used for emulsion-dipped preparations by lectin immunohistochemistry with *Bandeiraea simplicifolia* lectin I, isolectin B_4 (Laitinen, 1987). Cryostat sections (10 μ thick) were fixed in cold acetone, incubated overnight at 4°C with lectin in 10 mM HEPES buffer, 0.1 mM $CaCl_2$, pH 8.5, then immunostained by the avidin-biotin complex (ABC)-peroxidase method with diaminobenzidine development (Hsu *et al*., 1981).

Statistical analysis

Descriptive data are expressed as geometric means (95% confidence interval (95% CI) and between group comparisons were made by one way or repeated measures analysis of variance and Student's *t* test with Bonferroni correction by use of GraphPAD Instat (San Diego, U.S.A.). Equilibrium constants were derived from specific binding values by iterative non-linear regression assuming single site models with GraphPAD Inplot4 (San Diego, U.S.A.). Equilibrium binding densities (B_{eq}) were obtained with 0.25 nM [^{125}I]-(Sar^1, Ile^8)AII or 0.03 nM [^{125}I]-351A. Values for binding inhibition constants (K_i) and Hill coefficients (n_H) were derived by fitting to sigmoid curves.

Reagents

AII and (Sar^1, Ile^8)AII were purchased from Peninsula Laboratories (Merseyside, U.K.). Lisinopril was a gift from Dr A. Oldham (ZENECA Pharmaceuticals, Macclesfield, Cheshire, U.K.). The non-peptide AT_1 receptor antagonists, losartan (DuP 753, 2-n-butyl-4-chloro-5-(hydroxymethyl)-1-[2'(1H-tetrazol-5-yl)biphenyl-4-yl)-methyl]imidazole potassium) and DuP 532 (2-propyl-4-pentafluoroethyl-1-[2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]-imidazole-5-carboxylic acid) were gifts from Dr R.D. Smith and Dr T.M. Reilly, respectively (Du Pont Merck Pharmaceutical Co., Wilmington, Delaware, U.S.A.). The peptide AT_2 receptor ligand, CGP42112A (nicotine-Tyr- (N$^{\alpha}$benzyl- oxycarbonyl-Arg)Lys-His-Pro-Ile - OH) and the non-peptide AT_2 receptor antagonist, PD123319 (1(4-amino-3 -methylphenyl)methyl-5-diphenylacetyl - 4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid-2HCl), were kindly supplied by Dr M. de Gasparo (Ciba-Geigy Pharmaceuticals, Basle, Switzerland) and Dr J.A. Keiser (Parke-Davis, Ann Arbor, MI., U.S.A.). Sterile Dulbecco's PBS was obtained from Flow Laboratories (U.K.). [^{125}I]-(Sar^1, Ile^8)AII (specific activity 2200 Ci mmol^{-1}) and ^{133}Xe were obtained from DuPont-New England Nuclear Research Products (UK) Ltd. (Stevenage, U.K.) and DuPont Pharmaceuticals Ltd. (Letchworth, U.K.), respectively. 351A (N-[(s)-1-carboxy-3-phenylpropyl]-L-lysyl-tyrosyl-L-proline) was provided by Dr M. Hichens (Merck, Sharp and Dohme Laboratories, West Point, Pennsylvania). ^{125}I standards, and Hyperfilm-^{3}H were from Amersham International plc (Amersham, U.K.). Other unlabelled peptides, DTT, bacitracin, phosphoramidon, captopril, EDTA and enzyme free bovine serum albumin were from Sigma Chemical Co. (Poole, U.K.). *B. simplicifolia* lectin I, isolectin B_4, biotinylated antibodies and avidin-biotin complexes were from Vector Laboratories (Peterborough, U.K.).

Results

Effects of exogenous angiotensin receptor agonists on ^{133}Xe clearance and fibrovascular growth

In each experiment, ^{133}Xe clearance from PBS-injected sponges increased progressively from day 8 after implantation to reach a maximal level of 40 to 50% by day 14 (Figure 1). Daily administration AII (1 nmol per sponge day^{-1}) increased ^{133}Xe clearance and fibrovascular growth area in implanted sponges, compared to PBS-injected controls (Figures 1 and 2 and Table 1).

The increased ^{133}Xe clearance elicited by AII was completely inhibited by concurrent treatment with the non-subtype-selective AII receptor antagonist (Sar^1, Ile^8)AII (Figure 1a) and by the AT_1 receptor antagonists losartan and DuP 532 (Figure 1b), but not by the AT_2 receptor antagonist PD123319 (Figure 1c) (each used at 100 nmol per sponge day^{-1}). The AT_2 receptor agonist CGP42112A (100 nmol per sponge day^{-1}) did not significantly affect ^{133}Xe clearance, either when given alone or together with AII (Figure 1c). Losartan, but not PD123319, inhibited the increase in fibrovascular growth area induced by AII (Figure 2a–d, Table 1).

Effects of AT_1 and AT_2 receptor antagonists, angiotensin I and angiotensin converting enzyme inhibitors on ^{133}Xe clearance

The role of the endogenous angiotensin system in sponge neovascularization was investigated in the absence of exogenous angiotensin receptor agonist. In contrast to their actions in the presence of exogenous AII, daily administration of the AT_1 receptor antagonists, losartan and DuP 532, did not significantly affect ^{133}Xe clearance compared with PBS-treated controls in the absence of exogenous AII (Figure 1d). Similarly, (Sar^1, Ile^8)AII, or the AT_2 receptor antagonist PD123319 did not affect ^{133}Xe clearance in the absence of exogenous AII (data not shown). Local administration of AI (1 nmol per sponge

Table 1 Effect of angiotensin II antagonists on the increase in fibrovascular growth area induced by angiotension II in sponges 8 days after subcutaneous implantation in rats

Treatment group	n	*% fibrovascular growth area*
PBS	6	32 (28 to 35)
AII	6	62 (58 to 66)*
AII + losartan	6	34 (32 to 37)‡
AII + PD123319	4	57 (48 to 66)*

Daily injection of 1 nmol AII alone or with the AT_2 receptor antagonist PD123319 (100 nmol) increased the percentage of sponge occupied by fibrovascular tissue compared with PBS-injected controls (*$P < 0.001$). Daily injection of the AT_1 receptor antagonist losartan (100 nmol) together with AII reduced the fibrovascular growth area compared with sponges injected with AII alone (‡$P < 0.001$), to values not significantly different from PBS-injected controls. Values are means (95% CI).

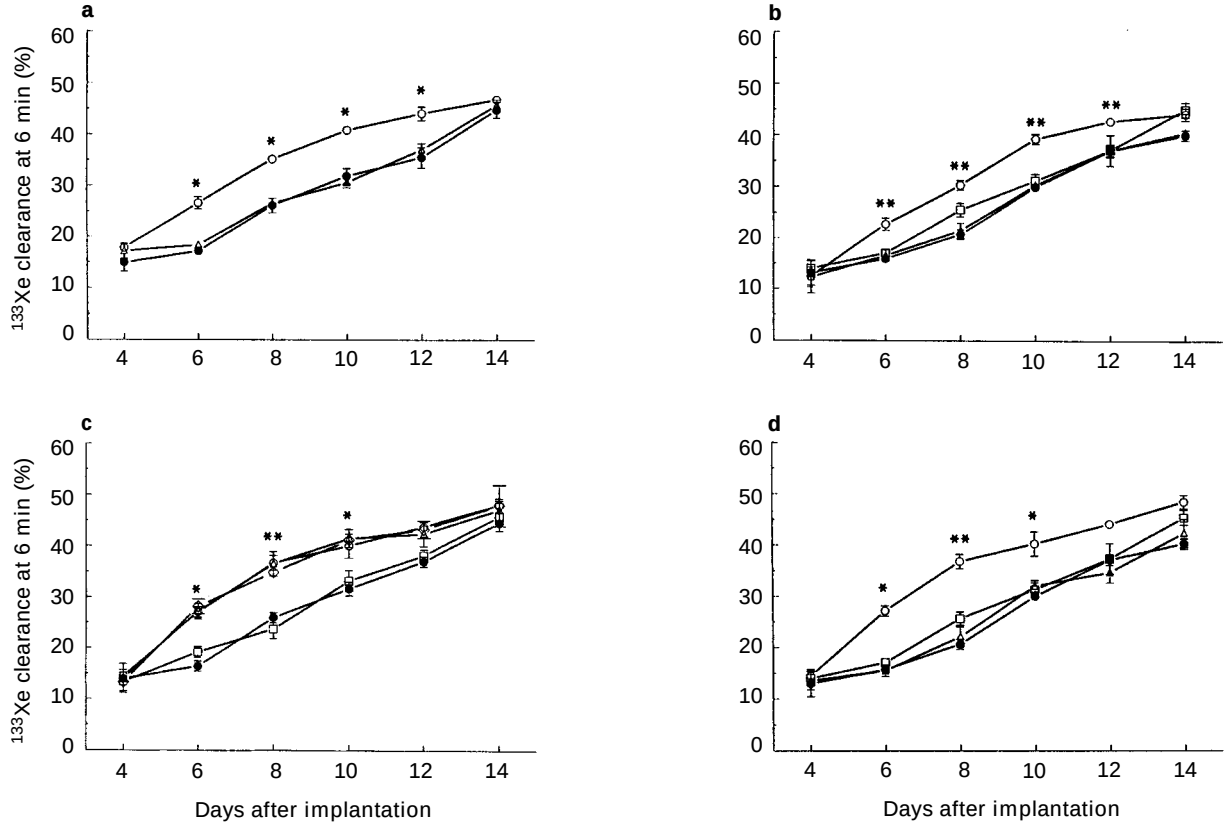

Figure 1 Effects of angiotensin receptor agonists and antagonists on ^{133}Xe clearance from implanted sponges. In each experiment, AII (○) enhanced ^{133}Xe clearance, compared with PBS-injected controls (●). AII was administered at 1 nmol per sponge day^{-1} and all other ligands at 100 nmol per sponge day^{-1}. (a) Inhibition of the AII-enhanced ^{133}Xe clearance by the non-subtype selective AII receptor antagonist (Sar^1, Ile^8)AII (△). (b) Inhibition of the AII-enhanced ^{133}Xe clearance by the AT_1 receptor antagonists, losartan (□) and DuP 532 (△). (c) Lack of inhibition of the AII-enhanced ^{133}Xe clearance (○) by the AT_2 receptor selective antagonist, PD123319 (△), and agonist, CGP42112A (◇). In the absence of exogenous AII, the AT_2 receptor selective agonist CGP42112A (□) did not affect ^{133}Xe clearance compared with PBS-injected controls (●). (d) Lack of effect on ^{133}Xe clearance of the AT_1 receptor antagonists, DuP 532 (△) and losartan (□), in the absence of exogenous AII. Points are means of 6 sponges and vertical lines show s.e.mean. *$P < 0.05$, **$P < 0.01$ for sponges receiving AII alone compared with a combination of AII and each antagonist (a and b), or for sponges receiving a combination of AII and PD123319 (c), or AII alone (d) compared with PBS-treated controls.

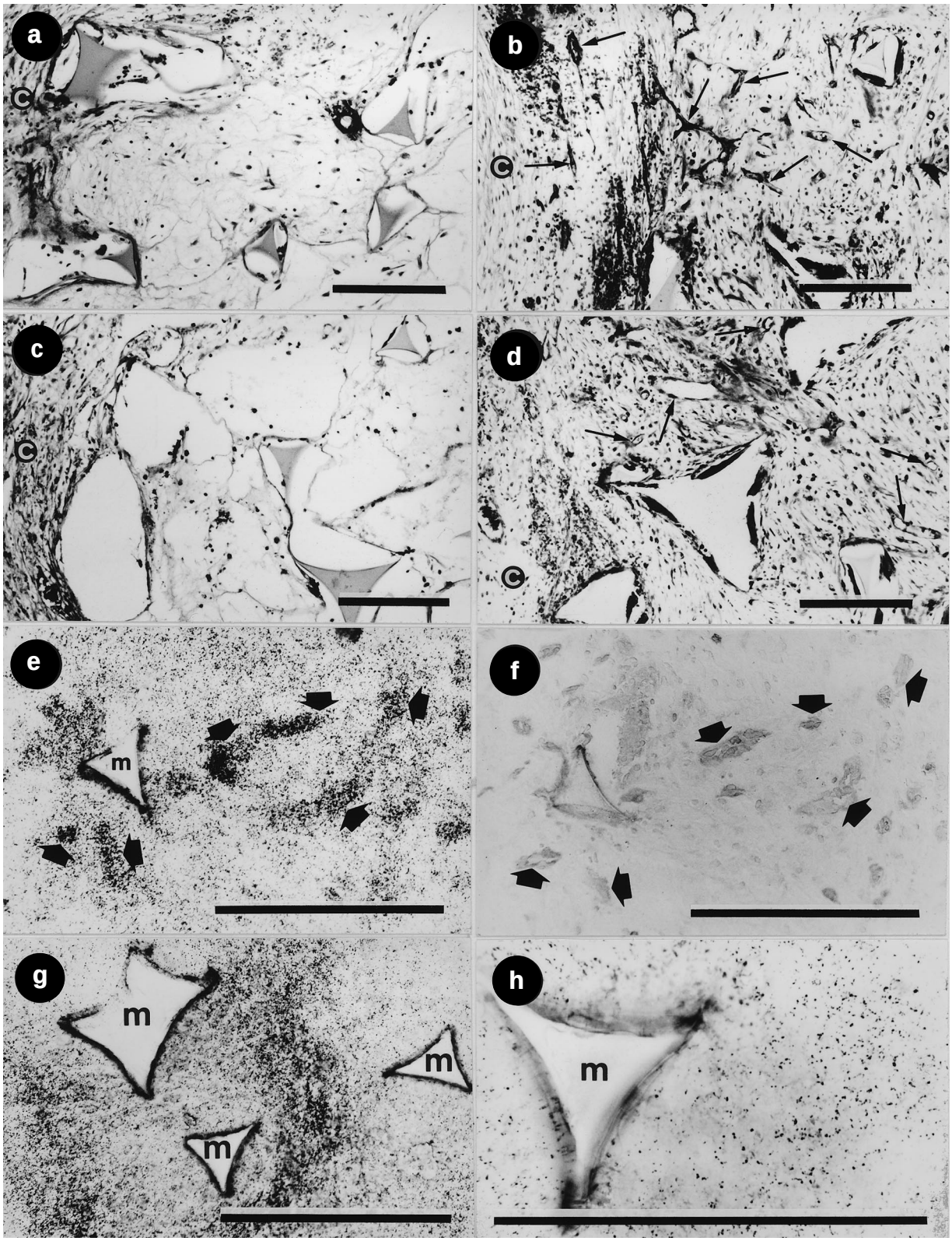

Figure 2 Microscopic localization of blood vessels (a–d, f) and [^{125}I]-(Sar^{1}, Ile^{8})AII binding (e, g, h) in sponges 8 days (a–d) or 14 days (e–h) after implantation. (a–d) Sponge granulomas treated by daily injection with PBS (a), angiotensin II (AII, 1 nmol day^{-1}) alone (b), AII plus 100 nmol day^{-1} losartan (c) or AII plus 100 nmol day^{-1} PD123319 (d). Sponges and surrounding tissue are illustrated from those regions in each section with the least fibrovascular ingrowth. Microvessels (fine arrows) are localized within tissue encapsulating the sponge and within the fibrovascular stroma infiltrating between elements of the sponge matrix. Daily injection with AII is associated with enhanced fibrovascular ingrowth and this is inhibited by the AT_1 receptor antagonist losartan, but not by the AT_2 receptor antagonist PD123319. (e) Dense [^{125}I]-(Sar^{1}, Ile^{8})AII binding to microvessels (broad arrows) within the sponge stroma, with less dense binding to stromal cells. (f) Consecutive section to (e) demonstrating microvascular endothelium. Arrows correspond to binding in (e). (g) [^{125}I]-(Sar^{1}, Ile^{8})AII binding to stroma between elements of sponge matrix, with less dense binding immediately adjacent to sponge matrix. (h) [^{125}I]-(Sar^{1}, Ile^{8})AII binding in the presence of 10 mM dithiothreitol to sponge stroma 14 days after implantation. Binding is to non-vascular stromal elements, with less dense binding immediately adjacent to the matrix. (a–d and f) Lectin immunohistochemistry for *Bandeiraea simplicifolia* lectin I, isolectin B_4, by use of avidin-biotin-peroxidase complex method. (e, g and h) Emulsion-dipped preparations. (a–e, g and h) Haematoxylin counterstain. c: surrounding tissue encapsulating the sponge, m: polyether sponge matrix. Bars = 200 μm.

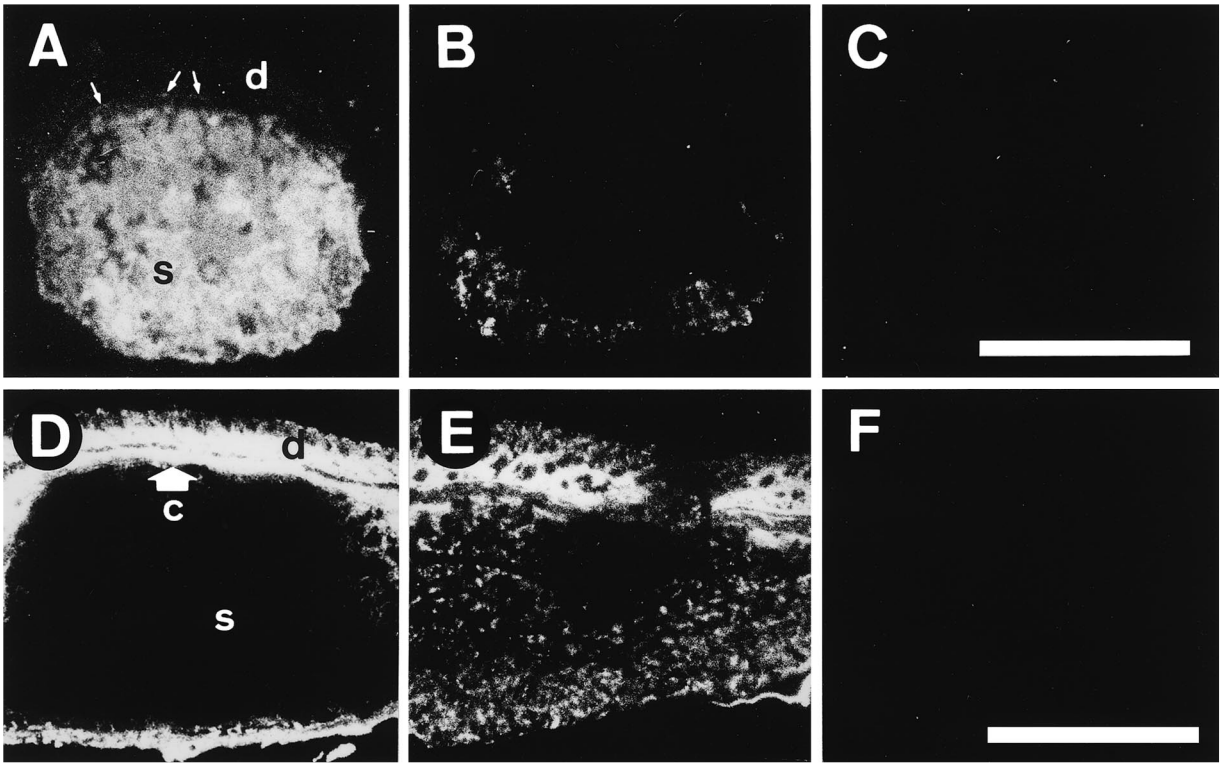

Figure 3 [^{125}I]-(Sar^1, Ile^8)AII (A–C) and [^{125}I]-351A (D–F) binding to PBS-treated rat sponge implants. (A) PBS-treated sponge 14 days after implantation demonstrating AT_1-like binding remaining in the presence of an excess (10 μM) of the non-radiolabelled selective AT_2 receptor antagonist PD123319. Binding was to sponge stroma (s) and to capsular blood vessels (arrows), with low density binding to overlying dermis (d). (B) AT_2-like binding in the presence of 10 mM dithiothreitol to a section consecutive to (A). Binding was to stromal elements within the sponge periphery. (C) Non-specific binding in the presene of an excess (1 μM) unlabelled (Sar^1, Ile^8)AII to a section consecutive to (B). (D) Binding of the angiotensin converting enzyme inhibitor [^{125}I]-351A to encapsulating tissue (c) and to the deep dermis (d) over a sponge 8 days after implantation, but not to sponge stroma (s). (E) [^{125}I]-351A binding to sponge stroma as well as capsule and dermis overlying a PBS-treated sponge 14 days after implantation. (F) Non-specific [^{125}I]-351A binding in the presence of 1 mM EDTA in a section consecutive to (E). Reversal prints of film autoradiograms. Bars = 5 mm.

day^{-1}), or of the ACE inhibitors captopril (1, 10 or 100 μg per sponge day^{-1}) or lisinopril (40 μg per sponge day^{-1}), or intraperitoneal administration of captopril or lisinopril (each at 100 mg kg^{-1} day^{-1}) did not significantly influence ^{133}Xe clearance compared with PBS-treated controls (data not shown).

Localization of [^{125}I]-(Sar^1, Ile^8)AII binding sites

Specific [^{125}I]-(Sar^1, Ile^8)AII binding sites were localized to microvessels (diameter < 100 μm) and stromal cells within the sponge, to microvessels in tissue surrounding the sponge and to dermis (Figures 2e–h and 3A–C). For each structure, specific binding represented $>90\%$ of total binding. [^{125}I]-(Sar^1, Ile^8)AII binding to sponge stroma was localized to cells between pieces of sponge matrix, with less dense binding to cells immediately adjacent to sponge matrix (Figure 2g). In the presence of 10 μM losartan or 10 mM DTT, specific microvascular and dermal [^{125}I]-(Sar^1, Ile^8)AII binding were abolished, as was most of the binding to the sponge stroma (Figures 2h and 3B). Low density specific binding to non-vascular stromal elements was localized between pieces of matrix in the presence of losartan or DTT (Figure 2h).

Sponges explanted at 4, 8 and 14 days revealed a progressive infiltration of the sponge stroma by both DTT-sensitive and DTT-resistant [^{125}I]-(Sar^1, Ile^8)AII binding sites (Figure 4). Surrounding tissue displayed microvascular DTT-sensitive, but not DTT-resistant sites (Figure 3A and B), with no difference in binding density between time points (data not shown). Both DTT-sensitive and -resistant sites were detected within stroma, near the sponge surface, at day 4, with no further increase in binding density in this region through day 14 (Figure 4a). DTT-sensitive sites in the central stroma progressively increased in density from day 4 to day 14, and DTT-resistant sites were first detected in the central stroma at day 14 (Figure 4b).

[^{125}I]-(Sar^1, Ile^8)AII binding to dermis overlying day 14 PBS-treated sponges ($B_{eq}=6.2$ (95% CI, 4.2 to 9.3) amol mm^{-2}) was of similar density to binding to dermis of normal skin ($B_{eq}=4.9$ (95% CI, 3.6 to 6.7) amol mm^{-2}, $P=0.44$). [^{125}I]-(Sar^1, Ile^8)AII binding sites were saturable and of high affinity in microvessels and sponge stroma (Table 2), and in dermis ($B_{max}=85$ (95% CI, 27 to 270) amol mm^{-2}, $K_d=5.0$ (95% CI, 1.8 to 13.7) nM)). [^{125}I]-(Sar^1, Ile^8)AII binding to sponge stroma and to blood vessels in tissue surrounding sponges had B_{max} values 5.5 and 3.8 times greater than did binding to overlying dermis ($P<0.01$ and $P<0.05$, respectively).

Characteristics of [^{125}I]-(Sar^1, Ile^8)AII binding sites

In the absence of DTT, stromal [^{125}]-(Sar^1, Ile^8)AII binding sites had similar characteristics to microvascular binding sites (Table 2). Binding was saturable with Hill coefficients approximating unity and was of high affinity (Table 2). Binding to microvessels and to stroma was inhibited with a rank order of affinity (Sar^1, Ile^8)AII $>$ AII $>$ losartan $\gg$ PD123319 (Table 2, Figure 5a). Specific binding to microvessels and to some regions of the sponge stroma was completely inhibited by 10 mM DTT and by 10 μM losartan (Table 2, Figures 3B and 4b).

Stromal [^{125}I]-(Sar^1, Ile^8)AII binding sites resistant to 10 mM DTT were an order of magnitude less dense than were specific binding sites in the absence of DTT (Table 2,, Figure 4a and b). DTT-resistant stromal binding sites were saturable,

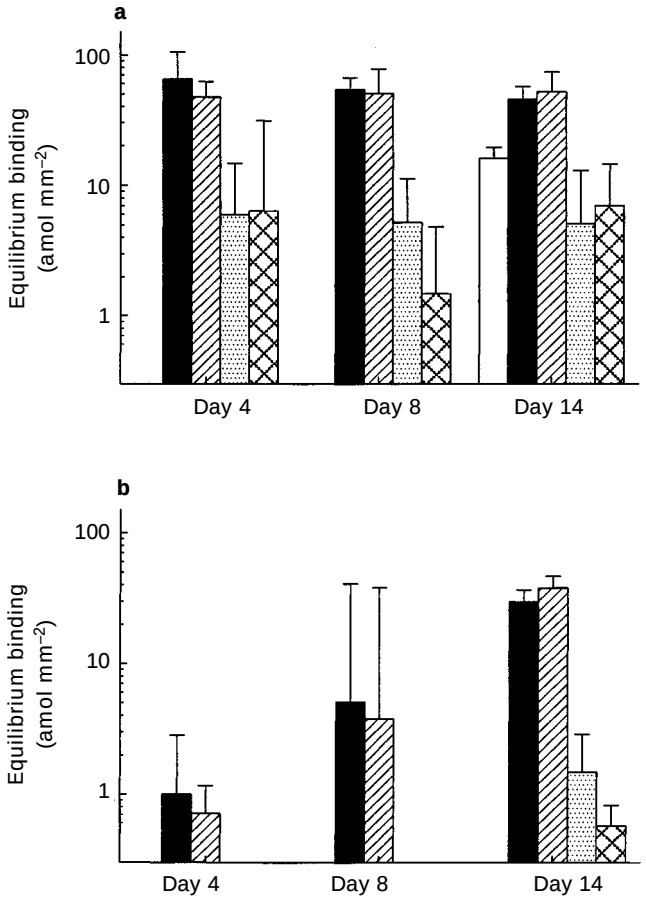

Figure 4 Development of [^{125}I]-(Sar^1, Ile^8)AII and [^{125}I]-351A binding sites in PBS-treated sponge implants. (a) Sponge stroma within 1.5 mm of the sponge surface. Specific [^{125}I]-351A binding sites were detected within sponges at day 14, but not at days 4 and 8. In contrast, [^{125}I]-(Sar^1, Ile^8)AII binding densities did not differ significantly between time points either in the presence of the AT_2 receptor antagonist PD123319 (10 μM) (AT_1 receptors remain unblocked), or in the presence of 10 μM losartan or 10 mM dithiothreitol (AT_2 receptors remain unblocked). (b) Central sponge stroma. PD123319-resistant [^{125}I]-(Sar^1, Ile^8)AII binding was more dense at day 14 than day 8 ($P<0.05$), and day 4 $P<0.01$). Specific losartan- and dithiothreitol-resistant [^{125}I]-(Sar^1, Ile^8)AII binding sites were detected at day 14, but not at earlier time points. Open columns, [^{125}I]-351A; solid columns, [^{125}I]-(Sar^1, Ile^8)AII; hatched columns [^{125}I]-(Sar^1, Ile^8)AII plus 10 μM PD123319 (AT_1 receptors remain unblocked); stippled columns, [^{125}I]-(Sar^1, Ile^8)AII plus 10 μM losartan or cross-hatched columns, 10 mM dithiothreitol (DTT) (AT_2 receptors remain unblocked). Columns represent geometric means ($\pm$95% CI) of 6 sponges. Analyses were performed on geometric data by one way analysis of variance and P values quoted after Bonferroni correction.

with a Hill coefficient of unity, and high affinity for (Sar^1, Ile^8)AII and a rank order of affinity (Sar^1, Ile^8)AII > AII > PD123319 $\gg$ losartan (Table 2, Figure 5b). In the presence of 10 μM losartan, [^{125}I]-(Sar^1, Ile^8)AII binding sites had a similar distribution and density to those in the presence of 10 mM DTT (Figure 4). Losartan-resistant specific binding was enhanced in the presence of 0.3 and 1 mM DTT by 71% (95% CI, 12% to 163%) and 71% (95% CI, 5% to 177%) respectively ($P<0.05$).

[^{125}I]-351A binding sites

[^{125}I]-351A bound specifically to the deep dermis (excluding hair follicles) and dermal blood vessels in normal rat skin and in skin overlying implanted sponges at all time points (Figure 3D–F). In addition, specific [^{125}I]-351A binding was localized to subdermal connective tissue in normal skin and to that surrounding sponges (Figure 3). Specific binding represented > 90% of total binding. Density of specific [^{125}I]-351A binding to dermal structures overlying sponges 4, 8 and 14 days after implantation did not differ significantly from binding densities in normal rat skin (data not shown). Specific [^{125}I]-351A binding was not observed within the stroma of PBS-treated sponges 4 and 8 days after implantation. On day 14, binding to sponge stroma was less dense than that to dermal structures (ratio B_{eq} sponge stroma : dermal vessels = 0.24 (95% CI, 0.15 to 0.39), $P=0.0006$).

Specific [^{125}I]-351-A binding to day 14 sponge granuloma and to dermal structures was completely inhibited in the presence of 1 mM EDTA by unlabelled lisinopril ($IC_{50}=1.8$ (95% CI, 1.0 to 3.1) nM) and by captopril ($IC_{50}=20$ (95% CI, 12 to 32) nM), but not by phosphoramidon (IC_{50} > 10 μM.

Disussion

As previously described, daily injection of 1 nmol AII enhanced ^{133}Xe clearance from subcutaneous sponge granulomas in rats, an effect which was inhibited by the selective AT_1 receptor antagonist losartan, but not by the AT_2 receptor antagonist PD123319 (Hu *et al*., 1996). Both of these effects were inhibited by losartan but not by PD123319, supporting our view that increased ^{133}Xe clearance is due to stimulation of angiogenesis by AII (Hu *et al*., 1996). Inhibition of AII-enhanced ^{133}Xe clearance by the selective AT_1 receptor antagonist, DuP532, and the lack of effect of the AT_2 receptor agonist, CGP42112A, further confirm that AII stimulates angiogenesis in this model via AT_1 receptors. [^{125}I]-(Sar^1, Ile^8)AII binding sites with characteristics of AT_1 receptors were localized to microvessels and stromal cells in sponge implants. We propose, therefore, that AII enhances angiogenesis in rat sponge granulomas by acting on AT_1 receptors within the granulation tissue.

The doubling by day 8 of fibrovascular growth area in sponges treated with AII is consistent with the increase in ^{133}Xe clearance above baseline being twice that observed in PBS-treated controls (Hu *et al*., 1995). The convergence of ^{133}Xe clearance measurements between AII- and PBS-treated sponges by day 14 is comparable to that observed with other angiogenic factors such as basic fibroblast growth factor (Hu *et al*., 1995). This could indicate that AII is angiogenic only during the early stages of granulation tissue formation, that other factors are generated at day 14 which inhibit AII-enhanced angiogenesis, or that the microvasculature becomes desensitized to the angiogenic effects of AII following chronic administration.

AII also has been implicated in angiogenesis in the absence of inflammation. It stimulates collateral vessel formation following arterial occlusion (Fernandez *et al*., 1982) and increases microvascular density in the rabbit cornea and rat cremaster muscle (Fernandez *et al*., 1985; Wang & Prewitt, 1990; Hernandez *et al*., 1992). Our data indicate that AII-enhanced angiogenesis in inflamed rat tissues is mediated by AT_1 receptors. Similarly, in non-inflamed rat cremaster muscle, AII-stimulated angiogenesis was inhibited by AT_1 receptor antagonists (Munzenmaier & Green, 1996). However, AII-induced angiogenesis in the chick chorioallantoic membrane was not inhibited by either AT_1 or AT_2 receptor antagonists, possibly reflecting different specificities of AII receptors in fowl compared with mammals (Le Noble *et al*., 1991; 1993; Nishimura *et al*., 1994).

Increased blood flow can stimulate angiogenesis and, under some circumstances, AT_1 receptor antagonists and ACE inhibitors can increase vascular density, possibly through vasodilatation-induced neovascularization (Unger *et al*., 1992; Maxfield *et al*., 1995; Sladek *et al*., 1996). However, some neovascular beds have a reduced vasoconstrictor response to AII (Andrade *et al*., 1992), and the balance between vasoconstriction-induced inhibition and AT_1 receptor-mediated stimulation of angiogenesis may differ between vascular beds

Table 2 Characteristics of [^{125}I]-(Sar^1, Ile^8) angiotensin II binding to rat sponge implants

	No dithiothreitol		*Dithiothreitol* 10 mM
	Vessels	*Stroma*	*Stroma*
	B_{max} (amol mm^{-2})		
	324 (190 to 553)	467 (314 to 693)	21 (10 to 44)*
	K_i (nM)		
(Sar^1, Ile^8)AII	3.4 (1.9 to 6.2)§	2.9 (2.1 to 4.2)‡	0.9 (0.4 to 2.2)#
AII	16 (6 to 1,121)‡	17 (13 to 22)‡	3.6 (1.4 to 9.3)‡
Losartan	635 (360 to 1,121)	468 (351 to 625)	> 10,000
PD123319	> 10,000	> 10,000	202 (108 to 376)
	n_H		
(Sar^1, Ile^8)AII	−1.0 (−0.7 to −1.3)	−1.0 (−0.9 to −1.2)	−1.0 (−0.7 to −1.3)
AII	−0.9 (−0.5 to −1.4)	−0.8 (−0.7 to −1.0)	−0.9 (−0.4 to −1.9)
Losartan	−0.9 (−0.7 to −1.2)	−0.8 (−0.6 to −1.0)	ND
PD123319	ND	ND	−1.0 (−0.7 to −1.4)

Maximal [^{125}I]-(Sar^1, Ile^8)AII binding to stroma was higher in the absence than in the presence of 10 nM dithiothreitol. For each structure, (Sar^1, Ile^8)AII had higher affinity than AII for [^{125}I]-(Sar^1, Ile^8)AII binding sites. In the absence of dithiothreitol, AII had higher affinity than losartan and in the presence of 10 mM dithiothreitol AII had higher affinity than PD123319. *$P < 0.0001$, ‡$P < 0.001$, §$P < 0.01$, #$P \leqslant 0.05$. Values are means (95% CI) of 6 cases. ND: not determined.

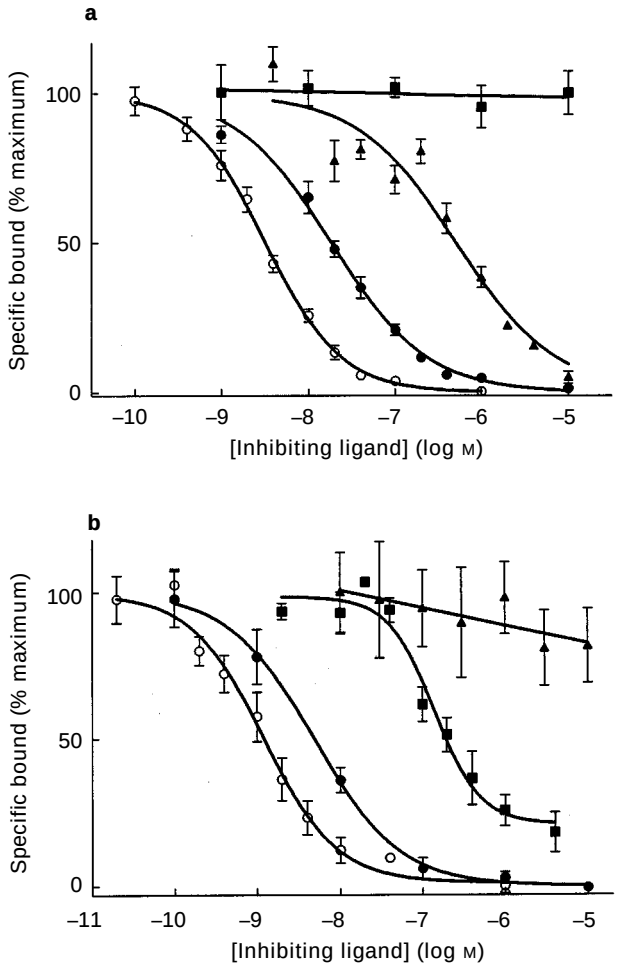

Figure 5 Characteristics of specific [^{125}I]-(Sar^1, Ile^8)AII binding to stroma of PBS-treated sponges 14 days after implantation. (a) Binding in the absence of dithiothreitol (DTT) had characteritics of AT_1 receptors, being inhibited by unlabelled (Sar^1, Ile^8)AII (○), angiotensin II (●) and losartan (▲), but not by PD123319 (■). (b) Binding in the presence of 10 mM DTT had characteristics of AT_2 receptors, being inhibited by unlabelled (Sar^1, Ile^8)AII (○), angiotensin II (●) and PD123319 (■), but not by losartan (▲). Points represent means of 5 to 6 sponges; vertical lines show s.e.mean.

and between disease states. Our data indicate that AT_1 receptor-mediated angiogenesis predominates in sponge granulomas.

AT_2 receptor agonists inhibit endothelial proliferation *in vitro* and the AT_2 receptor antagonist PD123319 potentiated the AII-stimulated increase in vascular density in rat cremaster muscle (Stoll *et al*., 1995; Munzenmaier & Greene, 1996). In contrast, PD123319 inhibited AII-enhanced endothelial cell migation *in vitro*, suggesting possible angiogenic effects of AT_2 receptor activation (Volpert *et al*., 1996). We found no evidence for an effect of AT_2 receptor activation on angiogenesis in sponge granulomas. AT_2 receptors are expressed by cultured endothelial cells (Stoll *et al*., 1995) but were not localized to microvessels in rat skin and sponge implants in the present study. Modulation of angiogenesis through AT_2 receptors may require their upregulation *in vivo*. AT_2 receptors are upregulated during wound healing and may be important in suppressing the angiogenic response after tissue repair (Viswanathan & Saavedra, 1992). Comparable with our findings in sponge granulomas, AT_2 receptor-like binding sites were not detected on microvessels in chronically inflamed human synovium (Walsh *et al*., 1994). Failure to express microvascular AT_2 receptors may be a feature of non-resolving tissue injury and could contribute to excessive angiogenesis in chronic inflammatory diseases such as human arthritis.

[^{125}I]-(Sar^1, Ile^8)AII binding sites had characteristics of receptors for AII, binding being specific, saturable, of high affinity and modulated by DTT (Timmermans *et al*., 1993). [^{125}I]-351A binding sites had characteristics of ACE, being inhibited by lisinopril and captopril and by EDTA, but not by the neutral endopeptidase inhibitor phosphoramidon (Mendelsohn, 1984; Correa *et al*., 1991; Sun *et al*., 1994). [^{125}I]-351A binds specifically to the active site of ACE and binding densities correlate with ACE activity in rat tissues (Correa *et al*., 1991). The distribution of AII receptors and ACE in rat skin were as previously described (Sun *et al*., 1994).

In sponge granulomas, AII receptors and ACE appeared sequentially during angiogenesis. AT_1 sites were localized throughout sponge stroma at day 4, whereas AT_2 sites were not observed in the centres of sponges until day 14 and ACE first appeared in sponge granulomas near their surfaces at day 14. Furthermore, AT_1 sites were more dense, whereas ACE was less dense within sponge stroma than in overlying skin at all time points. This sequential development of AII receptors before ACE in sponge granulomas is consistent with our functional data that exogenous AII, but not AI, stimulated angiogenesis between days 6 and 12. AII from distant sources

may reach microvascular AT_1 receptors via the circulation. However, our observation that AII receptor antagonists or ACE inhibitors did not influence basal angiogenesis in sponge granulomas in the absence of exogenous AII suggests that a local source of AII is necessary for stimulation of angiogenesis *in vivo*.

In contrast to our observations in sponges, ACE and AT_1 receptors co-localize to the microvasculature of chronically inflamed synovium from patients with rheumatoid arthritis (Walsh *et al*., 1994). In this circumstance, locally generated AII may contribute to vascular proliferation. The late appearance of ACE in sponge granulomas may reflect endothelial maturation, as observed *in vitro* (Shai *et al*., 1992). Development of receptors preceding that of a local source for their peptide ligand has also been found for substance P during angiogenesis in sponge granulomas (Walsh *et al*., 1996). Mismatch between sources and receptors for peptides may, therefore, indicate vascular immaturity, maturation requiring more than the 14 days studied in the sponge granuloma model.

AT_1 and AT_2 sites were localized to non-vascular cells which were not immediately adjacent to sponge matrix, a distribution which corresponds to that of myofibroblasts in sponge granulomas (Walsh *et al*., 1996). AT_1 sites on non-endothelial cells may contribute to the stimulation of angiogenesis by AII *in vivo* through the generation of paracrine agents such as basic fibroblast growth factor, platelet-derived growth factor and transforming growth factor β (Naftilan *et al*., 1990; Stouffer & Owens, 1992; Itoh *et al*., 1993). Non-vascular effects of AII on myofibroblast contraction, connective tissue growth and collagen synthesis may also be important in granuloma formation (Gabbiani *et al*., 1972; Sadoshima & Izumo, 1993; Brilla *et al*., 1994).

In conclusion, AII can stimulate angiogenesis, probably by acting on AT_1 receptors within the sponge granuloma. AII-enhanced angiogenesis requires a local source of AII, either administered experimentally, as in this study, or, we suggest, from locally expressed ACE. Vasoregulatory systems develop sequentially during vascular maturation, with AII receptors appearing before ACE, such that an immature microvasculature may not be subject to local regulation by endogenously generated AII. The role of AII in pathological angiogenesis will depend on the balance of expression of ACE, AT_1 and AT_2 receptors. In conditions such as human arthritis, where ACE and AT_1 receptors are co-localized to the microvasculature, and AT_2 receptors are absent, AII may stimulate excessive angiogenesis. Pharmacological manipulation of the balance between components of the AII systems may provide novel therapeutic tools in angiogenic diseases.

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