Angiotensin converting enzyme in human synovium: increased stromal $^{125}$I$^{351}$A binding in rheumatoid arthritis

David Andrew Walsh, John Catravas, John Wharton

Abstract

Objective—To determine whether tissue angiotensin converting enzyme (ACE) is increased in synovia from patients with rheumatoid arthritis, osteoarthritis or chondromalacia patellae.

Methods—Sections of synovia from patients with rheumatoid arthritis (n = 7), osteoarthritis (n = 7) or chondromalacia patellae (n = 6) were tested for immunoreactivity for ACE, and for binding of the ACE inhibitor $[^{125}]351$A. The amount of ACE was measured with computer assisted image analysis as the proportion of synovial section area occupied by ACE-immunoreactive cells, and the density of $[^{125}]351$A binding.

Results—$[^{125}]351$A binding sites had characteristics of ACE and colocalised with ACE-like immunoreactivity to microvascular endothelium and fibroblast-like stromal cells in inflamed and non-inflamed human synovium. Stromal $[^{125}]351$A binding densities (B$_S$) and the fraction of synovial section area occupied by ACE-immunoreactivity (fractional area) were higher in synovia from patients with rheumatoid arthritis ($B_S$ 28 amol/mm$^2$, fractional area 0.21) than from those with osteoarthritis ($B_S$ 9 amol/mm$^2$, fractional area 0.10) or chondromalacia patellae ($B_S$ 9 amol/mm$^2$, fractional area 0.09) (p < 0.05). Density of $[^{125}]351$A binding to stroma was similar to that to blood vessels in rheumatoid arthritis, but less dense than vascular binding in chondromalacia patellae and osteoarthritis. Increases in $[^{125}]351$A binding densities were attributable to increases in the numbers of binding sites, and were consistent with an increase in the density of ACE bearing stromal cells.

Conclusion—ACE is upregulated in synovial stroma in rheumatoid arthritis. Increased tissue ACE may result in increased local generation of the vasoconstrictor and mitogenic peptide angiotensin II and thereby potentiate synovial hyperplasia and proliferation in rheumatoid arthritis.

increased in vascular and stromal synovial compartments in human arthritis.

**Methods**

**PATIENTS**

Human synovium was collected at joint arthroplasty from patients with osteoarthritis or rheumatoid arthritis, diagnosed according to American Rheumatism Association criteria, and from patients undergoing carbon fibre resurfacing of articular cartilage for chondromalacia patellae. Patients with chondromalacia patellae had radiographically normal knees, with cartilage defects observed at arthroscopy.

Equilibrium $[^{[125]}]351A$ binding studies and ACE immunohistochemistry were performed on synovia from patients with chondromalacia patellae ($n = 6$, 6 male, median age $= 40$ years, range 22 to 60), osteoarthritis ($n = 7$, 4 male, median age $= 51$ years, range 40 to 70), or rheumatoid arthritis ($n = 7$, 2 male, median age $= 62$ years, range 51 to 74). $[^{[125]}]351A$ saturation studies and assessments of synovial cellularity were performed on synovia from patients with osteoarthritis ($n = 7$, 5 male, median age $= 66$ years, range 43 to 78), or rheumatoid arthritis ($n = 6$, 1 male, median age 61, range 51 to 68).

**TISSUE PREPARATION**

Tissue samples were embedded in Tissue-Tek (Miles Inc, Elkhart, Indiana, USA) and frozen to cork mounts in melting isopentane without prior fixation. Specimens were stored at $-70^\circ C$ until use. Ten $\mu$m thick sections were cut in a cryostat and thaw mounted onto Vectabond (Vector Laboratories, Peterborough, UK) treated slides, air dried and used immediately.

$[^{[125]}]351A$ BINDING

$[^{[125]}]351A$ was prepared as previously described by radiiodination of the tyrosyl group of 351A. $[^{[125]}]351A$ was purified to a specific activity of 2000 Ci/mmol by high performance liquid chromatography using a Waters C18 µBondapak analytical column utilising a mobile phase consisting of 88% 0.04 M phosphoric acid with triethylamine, pH 3 and 12% acetonitrile for 10 minutes, followed by a linear gradient from 12% to 25% acetonitrile over the next 40 minutes. The retention time of the monoiodinated product was 24 minutes. $[^{[125]}]351A$ was stored in the dark at 4$^\circ C$ until use.

Sections of synovium were preincubated for 10 minutes in 10 mM PBS, pH 7.4 (buffer A). Excess buffer was removed and sections were incubated for three hours with 0.03 nM $[^{[125]}]351A$ in buffer B (buffer A plus 0.2% bovine serum albumin). Non-specific binding was defined in the presence of 1 mM EDTA. After incubations, sections were washed twice for five minutes at 4$^\circ C$ in buffer A and rinsed in distilled water before being rapidly dried under a stream of cold air. Unless otherwise stated, all incubations were performed at 22$^\circ C$.

QUANTIFICATION OF RADIOLIGAND BINDING

Labelled sections were opposed to radiosensitive film (Hyperfilm-$^{3H}$, Amersham, UK) and exposed at 4$^\circ C$ for three to four days, or for five and 20 hours (saturation studies). Films were developed in Kodak D19 for three minutes at 15$^\circ C$ and autoradiographic images were analysed using a Symphny image analysis system (Seescan, Cambridge, UK). Tissue structures were segmented according to grey level, isolated by interactive thresholding then delineated interactively. Vascular $[^{[125]}]351A$ binding was discriminated from stromal binding in regions of synovium where the density of binding to vessels was greater than that to stroma, resulting in a mask of linear and annular features that corresponded to vessels in sections stained with haematoxylin and eosin or for ACE-like immunoreactivity (see below). Stromal binding was defined as binding to those regions of synovium that were excluded by this vascular mask. Binding density was derived from grey values by comparison with 10 $\mu$m thick $^{125}$Iodine standards (Amersham, UK) coexposed with each film, and corrected for the activity date of the ligand, assuming catastrophic decay. Equilibrium binding densities ($V_\text{max}$) and maximal binding capacities ($B_\text{max}$) are expressed as amount of bound ligand (amol) per mm$^2$ of blood vessel, or per mm$^2$ of stroma.

CHARACTERISATION OF BINDING SITES

Specific binding was defined as total minus non-specific binding. Equilibrium binding studies were performed on triplicate sections. Equilibrium binding density ($V_\text{max}$) was defined as the amount of $[^{[125]}]351A$ bound per unit area of the structure of interest after equilibration with an excess of 0.03 nM $[^{[125]}]351A$. Maximal binding capacities ($B_\text{max}$) and equilibrium dissociation constants ($K_\text{f}$) were calculated from saturation studies using 0.05 to 1 nM $[^{[125]}]351A$. $B_\text{max}$ was defined as the total number of binding sites per unit area of the structure of interest. $K_\text{f}$ was defined as the concentration of $[^{[125]}]351A$ leading to 50% occupancy of specific binding sites. Low $K_\text{f}$ values indicate high affinity for $[^{[125]}]351A$. Observed association rate constants ($k_{\text{on}}$), dissociation rate constants ($k_{\text{off}}$) and binding inhibition constants ($K_\text{I}$) were derived from studies performed on synovia from patients with rheumatoid arthritis. High $k_{\text{on}}$ values indicate a rapid rate of association of a given concentration of radioligand with its binding sites. High $K_\text{I}$ values indicate rapid dissociation of radioligand from its binding sites. Dissociation was assessed after incubation of sections with 0.03 nM $[^{[125]}]351A$ for 180 minutes followed by transfer of sections for a further 20 to 120 minutes to an excess of buffer A containing no radioligand. In inhibition studies, sections were incubated with 0.03 nM $[^{[125]}]351A$ together with 0 to 10 $\mu$M non-radiolabelled ligand. Low $K_\text{I}$ values indicate that a non-radiolabelled ligand has high affinity for the $[^{[125]}]351A$ binding site.

IMMUNOHISTOCHEMISTRY

Consecutive sections to those used in binding studies were used for immunolocalisation of ACE using the affinity purified rabbit...
polyclonal antihuman ACE antibody RH179. Cryostat sections (10 µm thick) were stained by the avidin-biotin complex (ABC) method. In brief, sections were fixed in cold acetone for five minutes at 4°C, incubated with primary antibody overnight at 4°C, then with biotinylated goat antirabbit IgG for 30 minutes, avidin-biotin-peroxidase complex for 30 minutes and finally developed in diaminobenzidine with glucose oxidase enhancement, dehydrated and mounted in dibutylpthalate polystyrene xylene (Raymond Lamb, London, UK).

QUANTIFICATION OF IMMUNOHISTOCHEMISTRY AND CELLULARITY

The fraction occupied by ACE immunoreactivity of synovial tissue section area within 200 µm of the synovial surface was measured using a KS300 image analysis system (Imaging Associates, Thame, UK). Using a × 20 objective lens, the three microscopic fields with the most intense stromal ACE immunoreactivity were selected per section. For each field, the synovial area of interest was delineated interactively, and the area occupied by ACE immunoreactivity was segmented according to grey level. ACE fractional area was defined as the sum of the ACE immunoreactive areas divided by the sum of the synovial areas.

Qualitative assessment of inflammation and quantification of cellularity were determined on haema-toxylin and eosin counterstained sections used in saturation studies described above. Using a × 10 objective lens, the three microscopic fields with the most intense cellularity were selected per section. For each field, the synovial area of interest was delineated interactively, and the area occupied by haema-toxylin positive nuclei was segmented according to hue. Nuclear fractional area was defined as the sum of the haema-toxylin positive areas divided by the sum of the synovial areas.

STATISTICAL ANALYSIS

Descriptive data are expressed as geometric (binding and cellularity data) or arithmetic (ACE fractional area) means (95% confidence intervals). Between group comparisons were made using SPSS for Windows release 6.1 statistical programme (SPSS Inc, Chicago, IL) by Student’s t test, or one way analysis of variance with post-hoc comparisons using Duncan’s multiple range test and a significance level set at 0.05. Correlations were determined using

<table>
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<th>Table 1 Characteristics of [125I]351A binding to human synovium</th>
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<tr>
<td>Kinetic studies</td>
</tr>
<tr>
<td>k_0 (s⁻¹)</td>
</tr>
<tr>
<td>k⁻¹ (s⁻¹)</td>
</tr>
<tr>
<td>K_i (nM)</td>
</tr>
<tr>
<td>Linopirul</td>
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<tr>
<td>Phosphoramidon</td>
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Values are means (95% CI) derived from synovia from six patients with rheumatoid arthritis. k_0 : observed association rate constant, k⁻¹ : dissociation rate constant, K_i : inhibition constant.
linear regression. The effect of introducing log10 nuclear fractional area as a covariate on the effect of diagnostic group on maximal binding capacity was determined using a general factorial model. Equilibrium constants were derived from specific binding values by iterative non-linear regression assuming single site models using GraphPAD Prism (San Diego, USA). Values for binding inhibition constants (Ki) were derived by fitting to sigmoid curves assuming competitive interaction.

REAGENTS
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, PA). [125I] standards, and Hyperfilm-3H were from Amersham International plc, Amersham, UK. Lisinopril, captopril and phosphoramidon, EDTA and enzyme free bovine serum albumin were from Sigma Chemical Co, Poole, UK. Polyclonal antibodies to human ACE were a kind gift of Dr A J Kenny, Membrane Peptidase Group, University of Leeds, UK. Biotinylated antibodies and avidin-biotin complexes were from Vector Laboratories, Peterborough, UK.

Results
[125I]351A binding sites had an identical distribution to ACE-LI both in inflamed and in non-inflamed synovium (fig 1). ACE-LI was localised to vascular endothelium and to stromal cells (fig 1). As previously described, stromal staining was localised to spindle shaped cells particularly those surrounding blood vessels and those immediately beneath the synovial lining cells.17 Both ACE-LI and [125I]351A binding appeared more dense to stromal cells in synovia from patients with rheumatoid arthritis, compared with osteoarthritis or chondromalacia patellae (fig 1).

Table 2 Density and affinity of [125I]351A binding to human synovium

<table>
<thead>
<tr>
<th></th>
<th>CMP (n=6)</th>
<th>OA (n=7)</th>
<th>RA (n=6–7)</th>
<th>CMP (n=6)</th>
<th>OA (n=7)</th>
<th>RA (n=6–7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beq (amol/mm²)</td>
<td>9(3 to 25)</td>
<td>9(4 to 23)</td>
<td>28(16 to 48)*</td>
<td>64(46 to 90)</td>
<td>50(37 to 66)</td>
<td>69(49 to 97)</td>
</tr>
<tr>
<td>Bmax (amol/mm²)</td>
<td>NA</td>
<td>118(61 to 229)</td>
<td>259(154 to 435)†</td>
<td>NA</td>
<td>380(188 to 769)‡</td>
<td>360(144 to 902)</td>
</tr>
<tr>
<td>Kd (nM)</td>
<td>NA</td>
<td>0.2(0.1 to 0.3)</td>
<td>0.3(0.2 to 0.4)</td>
<td>NA</td>
<td>0.1(0.1 to 0.2)</td>
<td>0.3(0.1 to 0.5)</td>
</tr>
</tbody>
</table>

*Significant heterogeneity between Beq values in stroma from patients with CMP, OA and RA (F=3.71, p=0.04). RA > OA and RA > CMP (p<0.05). †Bmax values for binding to stroma greater in RA than in OA (t=2.3, p=0.04). ‡Bmax values greater for binding to blood vessels than to stroma in OA (t=6.1, p=0.001), but not in RA. No significant differences in Kd values were found between disease groups. Binding to blood vessels did not differ significantly between disease groups. Values are means (95% CI). Abbreviations: Beq ; equilibrium binding density, Bmax ; maximal binding capacity, Kd ; equilibrium dissociation constant, NA ; not assessed, CMP ; chondromalacia patellae, OA ; osteoarthritis, RA ; rheumatoid arthritis.

Figure 2 Characteristics of [125I]351A binding to human synovium. (A) Association (filled circles) and dissociation (empty circles) at 22°C of 0.03 nM [125I]351A binding to blood vessels in synovia from patients with rheumatoid arthritis. For dissociation time course, sections were transferred to an excess of buffer without [125I]351A at 180 minutes (arrow). (B) Inhibition of 0.03 nM [125I]351A binding to blood vessels in synovia from patients with rheumatoid arthritis by unlabelled lisinopril (circles) or captopril (triangles). (C) Saturation of [125I]351A binding to blood vessels in synovia from patients with rheumatoid arthritis. (D) Saturation of [125I]351A binding to synovial stroma from patients with rheumatoid arthritis (filled circles) or osteoarthritis (empty circles). Points represent means (SEM) for synovia from six to eight patients.
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included some binding to vascular endothelium, and to fibroblast-like stromal cells, rather than to changes in ACE activity. Furthermore, the endothelial fractional areas (approximately 8%) determined in each of three disease groups in these studies were similar to ACE fractional areas in synovia from patients with chondromalacia patellae or osteoarthritis in this study, and less than half the values for ACE fractional areas in synovia from patients with rheumatoid arthritis. Qualitative analysis of synovial sections stained for ACE-like immunoreactivity further supported our interpretation that increased stromal \(^{[125]I}\)351A binding in rheumatoid arthritis reflects increased ACE on non-vascular cells (fig 1).

\[^{[125]I}\]351-A binding was saturable, of high affinity and reversible. It was completely inhibited by the zinc chelator EDTA, and by the specific ACE inhibitors lisinopril and captopril, but not by the neutral endopeptidase inhibitor phosphoramidon at up to 10 \(\mu\)M. These characteristics of \(^{[125]I}\)351A binding sites both on blood vessels and on stroma in human synovia were consistent with binding to the active site of ACE, which has previously been correlated with ACE activity.\(^{16,19}\) These findings indicate that the increased stromal \(^{[125]I}\)351A binding observed in synovia from patients with rheumatoid arthritis represents increased amounts of ACE.

Increased stromal ACE may indicate either an increase density of cells that express ACE, or an increase in ACE expression by stromal cells. Either of these interpretations of our data would be consistent with increased ACE activity in the synovia of patients with rheumatoid arthritis that may contribute to the raised ACE activity observed in synovial fluids from patients with rheumatoid arthritis.\(^{14,15}\) Cellularity was increased in synovia from patients with rheumatoid arthritis compared with those from patients with osteoarthritis, as indicated by an increased proportion of the section area occupied by haematoxylin positive nuclei. This increase in cellularity was sufficient to explain the increased \(^{[125]I}\)351A binding capacity in rheumatoid arthritis, supporting the suggestion that increased stromal ACE reflects an increased number of ACE bearing cells within the synovium. Clarification of whether individual cells express more ACE in synovia from patients with rheumatoid arthritis will require further work on primary cultures of synovial cells.

Increased ACE in tissue stroma has been observed in the heart after myocardial infarction and in hypertension, and in healing skin wounds.\(^{8,28}\) As in synovium, stromal ACE was localised to fibroblast-like cells in these tissues. Cultured fibroblasts and myofibroblasts inhibitory activity, resulting in reduced ACE activity within the tissue.
express ACE mRNA and protein, and display ACE-like enzymatic activity.8,9,10 ACE expression by fibroblast-like cells is generally lower than by endothelial cells.11 However, fibroblast expression of ACE is subject to upregulation by a variety of factors that are present in inflamed synovium, such as basic fibroblast growth factor.6

Serum concentrations of angiotensinogen, the precursor of ANG I, are increased, and synovial fluid renin activity is raised in rheumatoid arthritis.12,13 Increased concentrations both of ANG II precursors and of ACE led us to suggest that the synovial angiotensin system is activated in rheumatoid arthritis. Furthermore, AT₁ receptor binding and ACE colocalise in human synovium indicating that synovial AT₁ receptors are exposed to locally generated ANG II.14

ANG II that has been generated by stromal cells may have autocrine effects on fibroblasts. Synovial inflammation may be regarded as an abnormal repair process in which synovial proliferation leads to joint damage. ANG II, acting through AT₁ receptors, can stimulate fibroblast proliferation and the generation of matrix proteins and growth factors such as transforming growth factor β.15,16 ACE is upregulated on cardiac fibroblasts during the tissue repair that follows myocardial infarction.7 In this model, fibroblast proliferation is inhibited by specific ACE inhibitors and by AT₁ receptor antagonists, consistent with stimulatory roles for endogenous ACE and ANG II.17,18 Increased stromal ACE may contribute to synovial proliferation and hypertrophy in rheumatoid arthritis by increasing ANG II production within the stromal compartment.

ANG II produced by stromal cells may have paracrine actions on the synovial vasculature, in addition to autocrine effects on synovial fibroblast-like cells. ANG II is a potent vasoconstrictor, and can stimulate angiogenesis in inflamed tissues.19 Increased ANG II generation within inflamed synovium therefore may contribute to synovial hypoxia and angiogenesis, each of which has been implicated in the pathogenesis of persistent synovitis.20,21 In addition to its role in the renin-angiotensin system, ACE can inactivate other peptides including bradykinin and substance P.22 These peptides increase blood flow and plasma extravasation in acute models of inflammation, and may stimulate vascular and connective tissue proliferation in chronic inflammation.23,24 Synovial stromal cells and microvascular endothelium bear specific receptors for bradykinin and substance P respectively.25,26 and upregulation of synovial ACE may be important in reducing the pro-inflammatory activities of these peptides. The ACE inhibitor captopril had effects that were consistent with a slow acting anti-rheumatic agent in an open, prospective study of 15 patients with rheumatoid arthritis.27 ACE inhibition with pentopril, however, was not associated with clinical improvement in rheumatoid arthritis.28 These equivocal results may reflect the different roles of ACE in multiple regulatory peptide systems. In animal models, effects of ACE inhibition have sometimes been attributed to potentiation of kinins or substance P, rather than inhibition of ANG II generation.29,30 However, one mechanism of increased synovial ANG II generation in rheumatoid arthritis will require more selective intervention with agents such as the recently developed AT₁ receptor antagonists.31

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