Angiotensin converting enzyme in human synovium: increased stromal $^{125}$I351A binding in rheumatoid arthritis

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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 $[^{125}$I$]351A$. 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 $[^{125}$I$]351A$ binding.

Results—$[^{125}$I$]351A$ 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 $[^{125}$I$]351A$ binding densities ($B_n$) and the fraction of synovial section area occupied by ACE-immunoreactivity (fractional area) were higher in synovia from patients with rheumatoid arthritis ($B_n$ 28 amol/mm$^2$, fractional area 0.21) than from those with osteoarthritis ($B_n$ 9 amol/mm$^2$, fractional area 0.10) or chondromalacia patellae ($B_n$ 9 amol/mm$^2$, fractional area 0.09) ($p < 0.05$). Density of $[^{125}$I$]351A$ 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 $[^{125}$I$]351A$ 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 hypoxia and proliferation in rheumatoid arthritis.


Angiotensin converting enzyme (ACE, EC 3.4.15.1) is a membrane metallopeptidase that participates in tissue regulatory peptide systems involving angiotensin II (ANG II), bradykinin and substance P. ACE catalyses the formation of ANG II from its inactive precursor, ANG I, which itself is generated by cleavage of angiotensinogen by the protease renin. ANG II exerts its biological effects via specific, cell surface receptors, of which two major subtypes, named AT$_1$ and AT$_2$ receptors, have been identified in humans.

ANG II is a potent vasoconstrictor, and can stimulate angiogenesis, fibroblast proliferation, collagen synthesis and growth factor expression, each mediated by AT$_1$ receptors. ACE expression is upregulated by fibroblasts and myofibroblasts after tissue injury. Furthermore, ACE inhibitors and AT$_1$ receptor antagonists can inhibit interstitial hypertrophy, hyperplasia and angiogenesis in a variety of tissues.

Hypertrophy, hyperplasia and angiogenesis also are characteristic of persistent rheumatoid synovitis. ACE activity is raised in synovial fluids from patients with rheumatoid arthritis compared with those with osteoarthritis, and rheumatoid synovial explants release ACE activity into their culture medium indicating local synthesis. ACE, however, is primarily a membrane bound peptidase, and tissue ACE may be more important than soluble ACE in the local generation of ANG II. In human synovium, ACE-like immunoreactivity (ACE-LI) is localised both to vascular endothelium, and to fibroblast-like and some macrophage-like cells in the synovial stroma. It is currently unknown, however, whether tissue ACE is increased in either or both of these synovial compartments in rheumatoid arthritis. Increased tissue ACE could potentiate synovial hypertrophy and hyperplasia in rheumatoid arthritis through the increased generation of ANG II.

$[^{125}$I$]351A$, an iodotyrosyl derivative of the ACE inhibitor lisinopril, binds specifically to the active site of ACE, and the density of $[^{125}$I$]351A$ binding correlates closely with enzyme activity. Immunohistochemistry is a sensitive technique for localising ACE immunoreactivity and quantifying the proportion of tissue components expressing ACE. Quantitative in vitro receptor autoradiography permits the density of ACE-like binding sites to be measured and pharmacologically characterised. A combination of the two techniques performed on the same tissue samples provides a powerful tool to localise and quantify ACE at the microscopic level in human synovium.

We have now used quantitative in vitro autoradiography with $[^{125}$I$]351A$ and immunohistochemistry to test whether tissue ACE levels are...
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 \(^{125}\text{I}\)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). \(^{125}\text{I}\)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 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°C until use. Ten µm thick sections were cut in a cryostat and thaw mounted onto Vectabond (Vector Laboratories, Peterborough, UK) treated slides, air dried and used immediately.

\[^{125}\text{I}\]351A BINDING

\[^{125}\text{I}\]351A was prepared as previously described by radiiodination of the tyrosyl group of 351A. \[^{125}\text{I}\]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 containing no radioligand. In inhibition studies performed on synovia from patients with rheumatoid arthritis. High \(k_{\text{off}}\) values indicate a rapid rate of association of a given concentration of radioligand with its binding sites. High \(K_R\) values indicate rapid dissociation of radioligand from its binding sites. Dissociation was assessed after incubation of sections with 0.03 nM \[^{125}\text{I}\]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 \[^{125}\text{I}\]351A together with 0 to 10 µM non-radiolabelled ligand. Low \(K_R\) values indicate that a non-radiolabelled ligand has high affinity for the \[^{125}\text{I}\]351A binding site.

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 (\(B_\text{eq}\)) was defined as the amount of \[^{125}\text{I}\]351A bound per unit area of the structure of interest after equilibration with an excess of 0.03 nM \[^{125}\text{I}\]351A. Maximal binding capacities (\(B_\text{max}\)) and equilibrium dissociation constants (\(K_d\)) were calculated from saturation studies using 0.05 to 1 nM \[^{125}\text{I}\]351A. \(B_\text{max}\) was defined as the total number of binding sites per unit area of the structure of interest. \(K_d\) was defined as the concentration of \[^{125}\text{I}\]351A leading to 50% occupancy of specific binding sites. Low \(K_d\) values indicate high affinity for \[^{125}\text{I}\]351A. Observed association rate constants (\(k_{\text{on}}\)), dissociation rate constants (\(k_{\text{off}}\)) and binding inhibition constants (\(K_i\)) were derived from studies performed on synovia from patients with rheumatoid arthritis. High \(k_{\text{off}}\) values indicate a rapid rate of association of a given concentration of radioligand with its binding sites. High \(K_i\) values indicate rapid dissociation of radioligand from its binding sites. Dissociation was assessed after incubation of sections with 0.03 nM \[^{125}\text{I}\]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 \[^{125}\text{I}\]351A together with 0 to 10 µM non-radiolabelled ligand. Low \(K_i\) values indicate that a non-radiolabelled ligand has high affinity for the \[^{125}\text{I}\]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 RH179.24

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 dibutylphthalate 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 x 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.

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
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.

**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>Stroma</th>
<th>Blood vessels</th>
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<tbody>
<tr>
<td></td>
<td>CMP (n=6)</td>
<td>OA (n=7)</td>
</tr>
<tr>
<td>Beq (amol/mm²)</td>
<td>9 (3 to 25)</td>
<td>9 (4 to 23)</td>
</tr>
<tr>
<td>Bmax (amol/mm²)</td>
<td>NA</td>
<td>118 (61 to 229)</td>
</tr>
<tr>
<td>Kd (nM)</td>
<td>0.2 (0.1 to 0.3)</td>
<td>0.3 (0.2 to 0.4)</td>
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*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 (p=2.3, p=0.04). ‡Bmax values greater for binding to blood vessels than to stroma in OA (p=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.

**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).

[125I]351A binding to both vascular and non-vascular structures had characteristics of ACE (tables 1 and 2, fig 2). Binding was time dependent, reversible, saturable, and completely inhibited by the non-radiolabelled ACE inhibitor. Values for binding inhibition constants (Ki) were derived by fitting to sigmoid curves assuming competitive interaction.
inhibitors lisinopril and captopril, but not by the neutral endopeptidase inhibitor phosphoramidon (table 1). \[^{[125I]}351A\] binding was inhibited by 97% (95% CI, 93 to 98) in the presence of 1 mM EDTA.

Stromal equilibrium binding of \[^{[125I]}351A\] was higher in synovia from patients with rheumatoid arthritis than from those with osteoarthritis or chondromalacia patellae (table 2). Subsequent saturation studies confirmed the increased \[^{[125I]}351A\] binding to synovial stroma in rheumatoid arthritis, attributable to a twofold increase in the number of binding sites (Bmax), with no increase in their affinity (indicated by equilibrium dissociation constant (Kd)) (table 2, fig 2). Binding sites on blood vessels were more dense than on stroma in synovia from patients with osteoarthritis, whereas binding sites on blood vessels had a similar density to those in stroma in rheumatoid arthritis (table 2, fig 1).

The fraction of synovial section area occupied by ACE immunoreactivity differed between disease groups (F = 5.736, p = 0.012). ACE fractional area was greater in synovia from patients with rheumatoid arthritis (0.21 (95% CI, 0.14 to 0.28)), than in synovia from patients with osteoarthritis (0.10 (95% CI, 0.03 to 0.18), p < 0.05), and synovia from patients with chondromalacia patellae (0.09 (95% CI, 0.05 to 0.14), p < 0.05). ACE fractional area displayed positive correlation with log10 nuclear fractional area (r² = 0.41, F = 15.3, p = 0.001).

Four of six synovial samples from patients with rheumatoid arthritis, and two of seven samples from patients with osteoarthritis displayed perivascular accumulations of lymphocytes. The fraction of synovial section area occupied by haematoxylin positive nuclei was greater in samples from patients with rheumatoid arthritis (0.07 (95% CI, 0.05 to 0.10)), than in samples from patients with osteoarthritis (0.03 (95% CI, 0.02 to 0.06), p = 0.02).

log10 stromal \(^{[125I]}351A\) equilibrium binding density (r² = 0.32, F = 5.12, p = 0.04). Introduction of log10 nuclear fractional area as a covariate resulted in no significant difference in log10 stromal \[^{[125I]}351A\] maximal binding capacity being displayed between samples from patients with rheumatoid arthritis and those with osteoarthritis (F = 1.12, p = 0.32).

Discussion

Our findings indicate that tissue ACE levels are increased in synovia from patients with rheumatoid arthritis and that this increase is attributable to an increase in the density of ACE bearing stromal cells, rather than to changes in vascular ACE.

The distribution of \[^{[125I]}351A\] binding was identical to that of ACE-LI, confirming the localisation of ACE to synovial vascular endothelium, and to fibroblast-like stromal cells, particularly those localised immediately beneath the synovial lining cells.\(^{17}\) Stromal \[^{[125I]}351A\] binding, as defined in this study, included some binding to vascular endothelium, where vessels were small or displayed low density binding that did not allow their discrimination from stroma in the vascular mask. Differences in vascular densities between disease groups are unlikely to explain differences in stromal \[^{[125I]}351A\] binding because two parallel studies using immunoreactivity for CD34 and binding of Ulex europaeus agglutinin-I as endothelial markers did not indicate differences in the fraction of synovial section area occupied by endothelium between these disease groups.\(^{26}\) 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 \[^{[125I]}351A\] binding in rheumatoid arthritis reflects increased ACE on non-vascular cells (fig 1).

[^{[125I]}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 μM. These characteristics of \[^{[125I]}351A\] 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.\(^{24}\) These findings indicate that the increased stromal \[^{[125I]}351A\] binding observed in synovia from patients with rheumatoid arthritis represents increased amounts of ACE.

Increased stromal ACE may indicate either an increase in the 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 \[^{[125I]}351A\] 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.\(^{6}\) \(^{28}\) As in synovium, stromal ACE was localised to fibroblast-like cells in these tissues. Cultured fibroblasts and myofibroblasts
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regulatory peptide systems. In animal models, the precursor of ANG I, are increased, and syntovial fluid renin activity is raised in rheumatoid arthritis. Increased concentrations both of ANG II precursors and of ACE lead us to suggest that the syntovial angiotensin system is activated in rheumatoid arthritis. Furthermore, AT1 receptor binding and ACE colocalise in human synovium indicating that syntovial AT1 receptors are exposed to locally generated ANG II.

ANG II that has been generated by stromal cells may have autocrine effects on fibroblasts. Syntovial inflammation may be regarded as an abnormal repair process in which syntovial 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. ACE is upregulated on cardiac fibroblasts during the tissue repair that follows myocardial infarction. In this model, fibroblast proliferation is inhibited by specie ACE inhibitors and by AT1 receptor antagonists, consistent with stimulatory roles for endogenous ACE and ANG II.

Increased stromal ACE may contribute to syntovial 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 syntovial vasculature, in addition to autocrine effects on syntovial fibroblast-like cells. ANG II is a potent vasoconstrictor, and can stimulate angiogenesis in inflamed tissues. Increased ANG II generation within inflamed synovium therefore may contribute to syntovial hypoxia and angiogenesis, each of which has been implicated in the pathogenesis of persistent synovitis.

In addition to its role in the renin-angiotensin system, ACE can inactivate other peptides including bradykinin and substance P. These peptides increase blood flow and plasma extravasation in acute models of inflammation, and may stimulate vascular and connective tissue proliferation in chronic inflammation. Syntovial stromal cells and microvascular endothelium bear specific receptors for bradykinin and substance P respectively and upregulation of syntovial 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. ACE inhibition with pentopril, however, was not associated with clinical improvement in rheumatoid arthritis. 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. However, stimulation of the AT1 receptor by increased syntovial ANG II generation in rheumatoid arthritis will require more selective intervention with agents such as the recently developed AT1 receptor antagonists.


