Localisation of xanthine oxidase to synovial endothelium

C R Stevens, M Benboubetra, R Harrison, T Sahinoglu, E C Smith, D R Blake

Abstract

The presence of the xanthine oxidase enzyme system has been demonstrated in the rheumatoid synovium. This supplies a reactive oxygen species generating system to synovium that is subjected to hypoxic-reperfusion cycles (cf inflamed rheumatoid synovium). An antibody to bovine milk xanthine oxidase has been used to localise the enzyme by immunohistochemistry to synovial endothelium. This implicates the endothelial cell as playing a major part in exacerbations of synovitis, induced by radicals.

The inflammatory process leading to a persistent destructive arthritis clearly has multiple components. In 1986 we proposed that the peculiar persistence of inflammation within a diarthrodial joint might be explained by hypoxic reperfusion injury.¹ Such a mechanism has since been shown to occur.^{2,3} The 'injury' generated by hypoxic-reperfusion cycles is thought to be mediated predominantly by oxidative damage precipitated by reactive oxygen species, particularly the superoxide radical O_2^- , its dismutation product hydrogen peroxide (H_2O_2), and the highly cytotoxic hydroxyl radical. Our studies have confirmed that a variety of biomolecules, proteins, lipids, glycosaminoglycans can be damaged by oxidative modification, and that damage is amplified by reperfusion cycles induced by movement. Studies with electron spin resonance using spin traps suggest that at least one of the species, O_2^- and (H_2O_2 derived from it) can be generated by human rheumatoid synovium after ex vivo reperfusion cycles.⁴ The finding that low doses of oxypurinol, a xanthine oxidase inhibitor, limited radical production suggested an important role for the cytosolic xanthine oxidoreductase system. Previous studies have suggested that the synovium does indeed contain this enzyme.⁵ Our present study defines its cellular location.

Materials and methods

TISSUE PREPARATION

Samples of human synovium were obtained from rheumatoid joints at synovectomy during joint replacement and from normal joints at limb amputation in cases of osteosarcoma. Pieces of tissue up to 1 cm³ were oriented onto cork bases and snap frozen in isopentane-liquid nitrogen within one hour ex vivo. All specimens were stored at -70°C.

PREPARATION OF ANTIGEN (XANTHINE OXIDASE) FOR IMMUNISATION

Bovine milk xanthine oxidase purified from fresh cow's milk⁶ was applied to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and examined by both Coomassie brilliant blue and silver staining. This resulted in the appearance of one band at 150 kilodaltons (95%) and a minor band at 140 kilodaltons (5%). On a native gel (7.5% polyacrylamide) a single band of about 300 kilodaltons was obtained (Coomassie brilliant blue and silver stain). The ratio of protein to flavin groups (ratio of absorbance at 280 nm to absorbance at 450 nm) of the enzyme was found to be about 6 and enzymatic activity was 1.62 units/mg (81.4% was in the dehydrogenase form).

IMMUNISATION

Rabbits were immunised initially with complete Freund's adjuvant (400 µl) containing 200 µg of the immunising antigen (freshly prepared bovine milk xanthine oxidase). A similar injection in incomplete Freund's adjuvant was given on day 14, followed by injections of 200 µg of the xanthine oxidase in phosphate buffered saline (pH 7.3) on days 30 and 38. On day 36 a blood sample (10-15 ml/rabbit) was taken from the peripheral ear veins and the serum antibody level was assessed by enzyme linked immunosorbent assay (ELISA). Serum samples taken from rabbits before immunisation and after immunisation with bovine serum albumin were used as controls. The titres were confirmed by immunoblotting and dot-immunobinding assays. Rabbits were killed one week after the final injection and about 100 ml of serum was collected from each rabbit. The cross reactivity of anti-bovine milk xanthine oxidase with human antigen was shown by ELISA, using purified human xanthine oxidase⁷ as antigen and goat antirabbit IgG conjugated to horseradish peroxidase as secondary antibody.

IMMUNOHISTOCHEMISTRY

Frozen blocks of two normal and five rheumatoid synovium were sectioned to a thickness of about 6 µm. Sections were dried in air and fixed in cold acetone for five minutes. These preparations were stained using the Vectastain elite rabbit IgG ABC kit. The method uses an unlabelled primary antiserum (anti-bovine milk xanthine oxidase diluted 1:750), followed by a biotinylated secondary antibody and then a preformed avidin and biotinylated horseradish

London Inflammation Group, ARC Bone and Joint Research Unit, The London Hospital Medical College, London E1 2AD, UK

C R Stevens
T Sahinoglu
E C Smith
D R Blake

Biochemistry Department, University of Bath, Bath BA2 7AY, UK
M Benboubetra
R Harrison

Correspondence to:
Professor Blake.

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Figure 1 Rheumatoid synovium stained with antibody to bovine milk xanthine oxidase. Positively staining capillaries are indicated by arrows. Faint, non-specific intimal layer staining is indicated by asterisks.

Figure 2 Rheumatoid synovium stained with non-immune rabbit serum replacing primary antibody. Only faint non-specific intimal layer staining is apparent, indicated by the asterisks. Specific vascular staining is absent (arrows).

peroxidase macromolecular complex. Peroxidase binding sites were disclosed with 0.02% hydrogen peroxide and 0.1% diaminobenzidine tetrahydrochloride in 0.1 M TRIS buffered saline, pH 7.2. Sections were counterstained with Harris's haematoxylin, differentiated in acid alcohol, dehydrated, and mounted in DPX permanent mountant (BDH). Solutions containing no primary antibody and solutions containing non-immune rabbit serum (diluted 1:750) as the primary antibody, or anti-bovine milk xanthine oxidase (diluted 1:750) which had been adsorbed overnight with affinity purified bovine milk xanthine oxidase from the Biozyme Company, UK, were used as controls.

Results

Xanthine oxidoreductase antigen was found to be localised to capillary endothelium in all specimens of both normal and rheumatoid peripheral synovium. In larger vessels the endothelial staining was less intense and there seemed to be diffuse positive staining in the smooth muscle layers (fig 1). Synovium stained with anti-bovine milk xanthine oxidase also showed a slight staining effect in and around the lining layer. However, this effect was also seen in control specimens stained with non-immune rabbit serum (fig 2), though the specific vascular staining shown in fig 1 was absent. No staining could be achieved with antibody which had been adsorbed by purified antigen nor when the primary antibody was omitted. In addition, immunoreactivity was not detected on formalin fixed tissue nor on tissue which had been thawed and refrozen before sectioning.

Discussion

It is generally accepted that chemically reactive oxygen radical species are mediators of tissue injury in a variety of disease states. Much research has been specifically directed at ischaemia-reperfusion injury mediated by oxygen radicals. One of the mechanisms whereby postischaemic reperfusion can generate reactive oxygen species is dependent on the enzyme xanthine oxidase.⁸ The non-pathological dehydrogenase form of this enzyme oxidises hypoxanthine and xanthine to uric acid using NAD^+ as an electron acceptor. Under ischaemic conditions, however, this enzyme can be converted to an NAD^+ independent form which catalyses the same reaction using molecular oxygen as an electron acceptor, resulting in superoxide anion generation.^{9,10} We believe that this system is in operation during post-ischaemic reperfusion of rheumatoid synovium, contributing to the characteristic signs of radical attack present in synovial fluid. Our results confirm the presence of the enzyme in synovium and localise it to endothelium. The capillary endothelial location is in accordance with a previous sequence of immunohistochemical studies on a variety of human tissue, including heart, gut, and liver.¹¹ Interestingly, the therapeutic action of gold drugs in rheumatoid arthritis is thought to be through an endothelial cell antiproliferative mechanism.¹² Recent work suggests that this mechanism involves the modulation by gold of the conversion of xanthine dehydrogenase to xanthine oxidase.¹³

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