Partial purification and characterisation of a synovial fluid inhibitor of osteoblasts

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Abstract

A polypeptide inhibitor of osteoblast proliferation is described which occurs in synovial effusions of patients with rheumatoid arthritis. Partial purification of the inhibitor showed a molecular weight of approximately 81 000 by gel electrophoresis. This polypeptide seems to be unique as no inhibitor of osteoblasts of similar molecular weight has been previously described in rheumatoid synovial effusions.

Osteoporosis is well recognised in association with rheumatoid arthritis (RA). Generally it is regarded as being of two types, periarticular and generalised osteoporosis. The factors responsible for generalised osteoporosis in RA are multiple and include those resulting in decreased bone formation and enhanced bone loss. Assessment of bone disease in RA is made difficult by the effects, not only of the underlying disease, but also, as the disease progresses, of disuse due to loss of mobility, drug treatment, which may affect bone metabolism, and in some cases poor nutrition.¹ Juxta-articular loss of bone density may be evident within months after disease onset² and is recognised as a useful diagnostic feature of RA.³ This latter form of osteoporosis seems to be mediated by local disease mechanisms.⁴⁻⁶

Studies with isolated bone cell populations have found that many well characterised bone resorbing hormones have receptors on osteoblasts and affect osteoblast function.⁷ These hormones include parathyroid hormone, prostaglandin E, 1,25-dihydroxyvitamin D₃, and epidermal growth factor.⁸⁻¹⁰ Human tumour necrosis factors α and β secreted by monocyte-lymphocyte preparations have been found in bone organ cultures to stimulate bone resorption and inhibit bone formation.⁷ Fibroblast derived growth factors may also inhibit osteoblast function.¹¹ To learn more about the mechanisms of osteoporosis complicating RA the effect of synovial fluid on isolated osteoblasts was studied. A polypeptide which is inhibitory towards osteoblasts has been partially purified and is described below.

Patients and methods

PATIENTS

Synovial fluid was obtained from patients with definite or classical RA as determined by the American Rheumatism Association criteria.³ Fluids were only obtained during routine diagnostic or therapeutic procedures after informed consent had been given.

MATERIALS

Sephacryl S-200, Phastgels, and Phastgel buffer strips were obtained from Pharmacia, Uppsala, Sweden. Diethylaminoethyl (DEAE)-52 cellulose was purchased from Whatman Biochemicals, London. Blue-Sepharose was obtained from Bio-Rad, USA. Acrylamide, methylenebisacrylamide, and sodium dodecyl sulphate were obtained from BDH Chemicals, Poole, England. *N,N,N',N'*-Tetramethylenediamine and bovine serum albumin were purchased from Sigma Chemical Co, St Louis, Mo, USA. Medium 199 was obtained from Flow Laboratories, Irvine, Scotland and [methyl-³H]thymidine from Amersham, Buckinghamshire, UK. Collagenase (type II) was obtained from Worthington, Ohio, USA. All other chemicals were of the highest purity available.

METHODS

Isolation and culture of bone cells

Bone cell populations were isolated from the calvaria of 3-4 day old mice (Quackenbush) by a modification of a previously described method.¹² Briefly, the calvaria, consisting of frontal and parietal bones, were removed aseptically. They were incubated at 37°C with 5% CO₂ with gentle stirring in 5 ml of a sterile enzyme solution consisting of 0.4% collagenase (Worthington type II) and 0.1% bovine serum albumin (Sigma) made up with Medium 199. The initial digestion medium containing freed osteoclast bone cells was decanted and replaced with 5 ml of fresh enzyme solution after 10 minutes' incubation at 37°C with 5% CO₂. After the second 90 minute incubation the digestion medium containing freed osteoblast cells was collected. After centrifugation in Medium 199 (270 g, 10 minutes) the osteoblasts were counted in a Coulter counter (Coulter Electronics, Hialeah, Florida). The cells were resuspended in Medium 199 and plated at $1-1.5 \times 10^5$ cells/well in 96 well flat bottom plates (1.0 cm $\times$ 0.6 cm; Flow Laboratories). In some studies the rat osteosarcoma line UMR-106 was used.¹² Lineage of calvarial and UMR-106 cells was checked with alkaline phosphatase staining, and on all occasions dense staining for this enzyme was found.¹³

Controls

With each osteoblast assay appropriate controls were used, including resting cells, human albumin, AB serum, and buffers.

The cells were incubated for 24 hours at 37°C with 5% CO₂ before the samples at a 1:12 dilution were added. Osteoblast cultures were

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then incubated in quadruplicate with heat treated fetal calf serum together with the test sample. Heat treated fetal calf serum had been previously found to stimulate osteoblasts. After 48 hours of incubation the cultures were pulsed for 18 hours with 37 kBq of [methyl-³H]thymidine per well. The cells were harvested with a Skatron multisample harvester, and the incorporation of [methyl-³H]thymidine was determined by standard liquid scintillation counting techniques. Data were expressed as the percentage inhibition of heat treated fetal calf serum stimulated osteoblasts.

Ion exchange chromatography

Pooled synovial fluid was diluted with 50 mM TRIS-HCl buffer (pH 8.0) at 4°C and was applied to a 2.5 cm×30 cm column of DEAE-52 cellulose. The column was equilibrated with starting buffer—50 mM TRIS-HCl (pH 8.0). A linear gradient of 0 to 500 mM NaCl (volume=250 ml) was applied to the column at a flow of 84 ml/h. An LKB fraction collector (Bromma, Sweden) was used to assay fractions for osteoblast inhibition.

Gel filtration

The ion exchange fraction eluting between 1211 and 1225 ml (fraction P) was applied to a 2.5 cm×95 cm column of Sephacryl S-200 (Pharmacia) which was equilibrated with 50 mM TRIS-HCl buffer (pH 7.4) containing 100 mM NaCl. A flow of 45 ml/h was used and 9.0 ml fractions were collected. Fractions were assayed for osteoblast inhibition.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in a phosphate continuous buffer system (pH 7.4) was carried out as described by Weber and Osborn.¹⁴ Discontinuous PAGE was carried out by the method of Davis.¹⁵ Pharmacia Phastsystem was used to perform discontinuous PAGE and SDS-PAGE. Protein was eluted with TRIS buffer from PAGE gel slices and concentrated with

acetone as described by Hames¹⁶ before being applied to PAGE or SDS-PAGE. Molecular weights of marker proteins were phosphorylase b 94 000; albumin 66 500; ovalbumin 43 000; carbonic anhydrase 30 000; trypsin inhibitor 20 000; and lactalbumin 14 000. The gels were stained with a silver stain using the method of Merril *et al.*¹⁷

Blue-Sepharose affinity chromatography

Blue-Sepharose was equilibrated with six changes of starting buffer—50 mM TRIS-HCl buffer (pH 7.0) containing 100 mM KCl. After six washes in buffer and centrifuging at 1230 g for 10 minutes the synovial fluid was diluted 1:2 with starting buffer before being applied to an equal volume of Blue-Sepharose to form a slurry. After six washes with starting buffer by centrifuging at 1230 g for five minutes the bound components were eluted with elution buffer—50 mM TRIS-HCl buffer (pH 7.0) with 1500 mM KCl. Aliquots of unbound and bound fractions were dialysed and tested for osteoblast inhibition.

Results

CONTROLS

There was no significant inhibition of tritiated thymidine incorporation (this ranged from 0 to 15% in the many assays performed) or evidence of morphological change by light microscopy with any of the controls used in the osteoblast assay.

ION EXCHANGE CHROMATOGRAPHY

Maximal osteoblast inhibition occurred in fraction P which eluted between 1211 and 1225 ml (fig 1).

GEL FILTRATION

Fraction D eluting between 245 and 259 ml showed the greatest osteoblast inhibition (fig 2).

GEL ELECTROPHORESIS

Ion exchange and Sephacryl S-200 fractions P and D respectively were subjected to SDS-PAGE in the presence of 2-mercaptoethanol, and the gels were stained with silver nitrate. The ion exchange fraction P contained nine major bands and several minor bands. The bands were of various molecular weights. Some were high molecular weight, several bands were similar in molecular weight to albumin (66 500), and three bands were in the low molecular weight region with molecular weights of 30 000, 20 000, and 14 000 (fig 3a, column P). Sephacryl S-200 fraction D contained five bands. These included two fine bands, which appeared to comigrate with human serum albumin and prealbumin, and a third band with a molecular weight of 81 000, which was also one of the bands observed in fraction P (fig 3a, column D).

Unfractionated synovial fluid, ion exchange fraction P, and Sephacryl S-200 fraction D were subjected to PAGE. Synovial fluid and the ion exchange fraction were found to contain several

Figure 1: Ion exchange chromatography of pooled synovial fluid 8.3 ml, diluted 1:20 with 50 mM TRIS-HCl buffer (pH 8.0), DEAE column (2.5 cm×30 cm) equilibrated with starting buffer, 50 mM TRIS-HCl buffer (pH 8.0), eluted with a linear gradient of NaCl concentration (0.5 mol/l) 250 ml. Temperature 4°C. Fraction volume 14 ml. ●=A₂₈₀; ○=percentage inhibition; ×.....×=salt gradient.

Figure 2: Sephacryl S-200 chromatography of pooled ion exchange fraction P. Column (2.5 cm × 95 cm) equilibrated with 50 mM TRIS-HCl buffer (pH 7.4), containing 100 mM NaCl. Temperature 4°C. Fraction volume 14 ml. ● = A_{280} ; ○ = percentage inhibition.

bands (fig 3b, columns SF and P respectively). Sephacryl S-200 fraction D contained one band with silver staining (fig 3b, column D).

BLUE-SEPHAROSE AFFINITY CHROMATOGRAPHY
Synovial fluid was subjected to Blue-Sepharose affinity chromatography and wash and elution fractions were assayed for osteoblast inhibition. Maximum inhibition was observed in the wash fractions and no inhibition was observed in the eluted fractions. The washed fraction was then subjected to PAGE and the different bands cut out. Protein was eluted from the gel slices by diffusion and assayed for osteoblast inhibition. Most inhibition occurred in one gel slice. When this gel slice was subjected to SDS-PAGE in the presence of 2-mercaptoethanol three visible

bands were seen with the middle band migrating the same distance as albumin (fig 4a, column A). With PAGE one band was found with silver staining (fig 4b, column B).

The table shows the results of the two purification sequences and indicates similar improvements in specific activity over the starting synovial fluid samples.

Light microscopic assessment showed that at least 80% of cells rounded up and developed an intracellular granular appearance and would no longer take up tritiated thymidine after culture with the inhibitor. These appearances were observed with both calvarial osteoblasts and UMR-106 cells. An S-200 enriched preparation of the inhibitor did not cause calvarial cell death as judged by trypan blue dye exclusion.

Discussion

This is the first report of an inhibitor of

Purification table

Material	Protein (g/l)	% Inhibition	Specific activity
Pooled synovial fluid	110	8	3.6
DEAE*52 fraction P	1.17	95	4 060
Sephacryl S-200 fraction D	0.052	98	98 000
Starting synovial fluid	17.9	44	123
Blue-Sepharose washed fraction	1.2	49	2 042
PAGE* gel slice	0.05	99	99 000

*DEAE=diethylaminoethyl; PAGE=polyacrylamide gel electrophoresis.

The starting synovial fluid was selected after prior assaying to ensure significant inhibition. In the first purification sequence pooled synovial fluid (n=3) was used, whereas in the second sequence fluid from a single patient was used. Albumin showed some inhibition of tritiated thymidine incorporation by osteoblasts, but on a weight for weight basis the inhibitor was over 720 times more active.

Figure 3: (a) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of 2-mercaptoethanol of D=the Sephacryl S-200 fraction D (▲ indicates the 81 000 molecular weight band); P=ion exchange fraction P; M=marker proteins of known molecular weight. (b) Discontinuous PAGE of D=Sephacryl S-200 fraction D (▲ indicates the single band); P=ion exchange fraction P; SF=synovial fluid. The gels were stained with silver nitrate.

Figure 4: (a) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of 2-mercaptoethanol of A=PAGE gel slice (▶ indicates the 81 000 molecular weight band); B=Blue-Sepharose washed fraction; M=marker proteins of known molecular weight. (b) Discontinuous PAGE of A=Blue-Sepharose washed fraction; B=gel slice with maximal osteoblast inhibition (▶ indicates the single band). The gels were stained with silver nitrate.

osteoblasts described in rheumatoid synovial fluid. The inhibitor significantly decreased tritiated thymidine incorporation of both murine osteoblast and rat osteosarcoma cells and microscopically resulted in morphological changes during culture. The inhibitor is probably a polypeptide of molecular weight 81 000, which under reducing and denaturing conditions consists of two separate bands on gel electrophoresis (figs 3a and b). Known inhibitors of osteoblast function, including tumour necrosis factors α and β (previously called cachectin and lymphotoxin respectively), have been shown to inhibit rat bone collagen and non-collagen protein synthesis in vitro.¹⁸ In our report osteoblast inhibitory activity was measured by the incorporation of tritiated thymidine and therefore our results may not be directly comparable with the action of tumour necrosis factor on osteoblasts.

The separation of the inhibitor with Blue-Sepharose was undertaken because of the high concentration of albumin in all fluids studied. Albumin was found to inhibit osteoblasts at high concentration only, with the inhibitor being over 720 times more active on a weight for weight basis. Both purification procedures gave similar improvements in specific activity over starting samples (table). Based on a molecular weight of 81 000 the inhibitor was active at a concentration of 3.3 $\mu\text{mol/l}$ or less.

Studies showed that 57% (17/30) of synovial fluid samples from patients with RA inhibited osteoblast proliferation. At present the biological role of this inhibitor requires further investigation. The possible mechanism of action is uncertain, though according to trypan blue dye exclusion the inhibitor is not toxic to mouse calvarial cells.

This inhibitor could be responsible for the periarticular osteoporosis often observed in RA. It appears different from previous inhibitors, such as fibroblast growth factors and the tumour

necrosis factors α and β . Although fibroblast growth factors increase the number of osteoblastic cells, they may under some conditions directly inhibit osteoblastic function.¹¹ Tumour necrosis factors α and β show time dependent dual stimulatory and inhibitory effects on bone formation.^{19 20} The molecular weights of these inhibitors are much less than that of the inhibitor found in RA synovial fluid, suggesting that the latter is unique.

The above is a preliminary report of the partial purification of a polypeptide inhibitor of osteoblast proliferation found in rheumatoid synovial effusions. Further work is aimed at defining the structure and function of this inhibitor.

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