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Human synovial cells secrete a 39 kDa protein similar to a bovine mammary protein expressed during the non-lactating period

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By SDS/PAGE analysis we have observed that human synovial cell monolayers secrete a prominent 39 kDa protein which could not be detected in skin and lung fibroblasts. This protein was purified to homogeneity by heparin–Sepharose chromatography and reverse-phase h.p.l.c. The *N*-terminal sequence was found to be almost identical to that of a recently described bovine protein detected in the mammary secretions during the involutionary phase of the lactational cycle. Characterization of this 39 kDa protein may provide a useful marker for classification of connective tissue cells.

INTRODUCTION

The articular cavity of joints is lined by a membrane (the synovial membrane) which consists of between one and three layers of synovial cells. The majority of these lining cells are fibroblast-like cells and are often referred to as synovial fibroblasts. However, differences exist between synovial cells and, for example, skin and lung fibroblasts. In addition to unique morphological characteristics [1], synovial cells proliferate more slowly [2,3], and we have also observed that synovial cells secrete proteins which are both qualitatively and quantitatively different from those secreted by lung and skin fibroblasts [4]. The most striking difference between synovial cells and other fibroblasts is the constitutive secretion of a prominent 39 kDa protein by synovial cells. During our attempts to purify the proteins secreted by synovial cells we observed that the 39 kDa protein binds to heparin. This observation made it possible to develop an effective purification procedure and to determine the *N*-terminal sequence of the first 24 residues of the secreted form. The *N*-terminal sequence of this 39 kDa material is almost identical to that of a glycoprotein isolated recently from mammary secretions of non-lactating cows. With the exception of this bovine glycoprotein, no significant similarity to any reported amino acid sequence was found.

MATERIALS AND METHODS

Cell lines

Synovial cells were derived by proteolytic digestion from the tissue of osteoarthritic and rheumatoid arthritic patients undergoing joint replacement [5] and were used between the 4th and 22nd passages. Apparently normal fetal lung (HFL1) and progressive systemic sclerosis (PSS) skin fibroblasts (CRL1108) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Apparently normal fetal (GM0010) and aged (AG4148) skin fibroblasts were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ, U.S.A.). Fibroblasts from Bloom syndrome, Fanconi anaemia and Cockayne syndrome patients were also obtained from this cell repository. The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, U.S.A.) containing 10% fetal calf serum.

SDS/polyacrylamide-gel electrophoresis

Cells were plated out at 5×10^4 cells/cm^2 in multiwell plates (Costar, Cambridge, MA, U.S.A.) in DMEM containing 10% heat-inactivated fetal calf serum. After 3 days, the monolayers were rinsed extensively to remove serum and incubated in minimum essential medium. Recombinant human β-interleukin 1 (IL-1) (10^7 units/mg; Cistron, Pine Brook, NJ, U.S.A.) or recombinant human tumour necrosis factor α (TNFα; Genzyme) was added. After 24–48 h the supernatants were collected, dialysed extensively, lyophilized and solubilized in an appropriate volume of sample buffer. An amount corresponding to 1 ml of culture medium was analysed on a 7.5–15% gradient polyacrylamide gel in the presence of SDS [6]. The gels were stained with Coomassie Brilliant Blue and/or silver stain (Silver Stain Kit, Bio-Rad). [^{35}S]Cys and [^{35}S]Met labelling experiments were conducted in a similar manner, except that the cells were incubated in cysteine-free or methionine-free medium supplemented with the respective labelled amino acids. Following electrophoresis, gels were dried and exposed to X-Omat AR film (Eastman–Kodak) for autoradiography.

Collection of secreted proteins

The synovial cells were plated at 4×10^4 cells/cm^2 in 75 cm^2 flasks (Corning Glass Works, Corning, NY, U.S.A.) using 10 ml of medium/flask, and were maintained for 2–4 weeks in DMEM containing 10% fetal calf serum. For the production of culture media the cells were extensively rinsed to remove as much serum protein as possible. The cells were incubated for 48 h in DMEM, then the supernatant was removed and concentrated, and a sample of the collected medium was analysed on an SDS/polyacrylamide gradient gel to estimate the amount of protein secreted and to check the effectiveness of the serum removal [4]. For radiosequencing, the cells were handled in a similar manner, except that the cells were maintained in cysteine-free medium supplemented with [^{35}S]Cys.

Purification

The culture media were concentrated and dialysed on an ultrafiltration device using a 10 kDa molecular mass cut-off filter (YM10; Amicon Corporation, Danvers, MA, U.S.A.). After dialysis to the starting buffer (0.05 M-NaCl/0.01 M-phosphate

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; IL-1, interleukin 1; TNF, tumour necrosis factor; PSS, progressive systemic sclerosis.

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buffer, pH 7.0) the concentrated medium was applied to a heparin–Sepharose CL-6B column (Pharmacia AB, Uppsala, Sweden; bed volume 10 ml) at a flow rate of 5 ml/h. The column was washed with 3 column vol. of the starting buffer and eluted with a 100 ml NaCl gradient (0.05–1.2 M) at a flow rate of 10 ml/h. Fractions of 5 ml were collected.

Fractions from the heparin–Sepharose chromatography corresponding to 0.5–0.9 M-NaCl were pooled and applied to a C_{18} reverse-phase column (Millipore/Waters), and the proteins were eluted with a 0–60% acetonitrile linear gradient in 0.1% trifluoroacetate over a 60 min period at 1 ml/min. Fractions of 2 ml were collected, dried on a centrifugal concentrator, solubilized in sample buffer and an amount corresponding to 0.1 ml of the fraction was analysed by SDS/PAGE.

Sequence analysis

The fractions from the heparin–Sepharose column corresponding to 0.5–0.9 M-NaCl were pooled, concentrated and dried on Sep-Pak C_{18} cartridges according to the manufacturer's recommendations (Millipore/Waters Associates, Milford, MA, U.S.A.). The dried material was solubilized in sample buffer, subjected to SDS/PAGE under reducing conditions, blotted on to a polyvinylidene difluoride membrane (Millipore) [7] and stained with Coomassie Brilliant Blue. The 39 kDa band (approx. 20 μg) was excised and subjected to sequential Edman degradation on a gas phase sequencer (Applied Biosystems 120A analyser). The position of the cysteine residue (Cys-5) was determined by radiosequencing. The sequence was read independently by two persons. The first 12 residues were confirmed in three independent runs from two synovial cell lines. Radiosequencing of the first 16 residues was performed in one case, using [^{35}S]Cys-labelled protein. The labelled proteins in the medium were extensively dialysed against 0.05 M-ammonium acetate, freeze-dried, solubilized in sample buffer, separated on SDS/PAGE and blotted. The 39 kDa band was excised and applied to the sequencer. The β-radiation of the fractions eluting from the on-line analyser was measured.

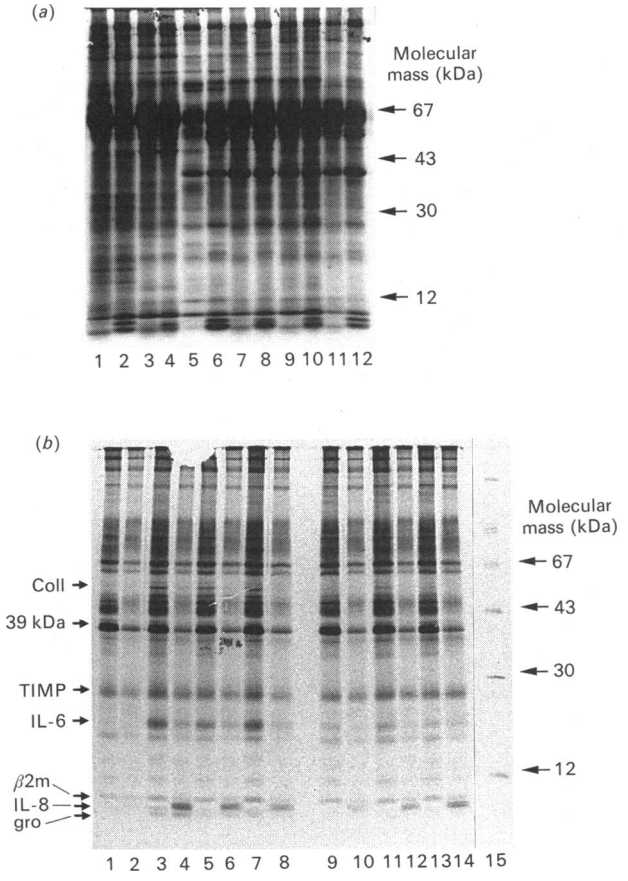

Fig. 1. SDS/PAGE analysis of secreted proteins

(*a*) Coomassie Brilliant Blue staining. Cell lines: lanes 1 and 2, HFL; 3 and 4, AG4148; 5 and 6, SC177; 7 and 8, SC186; 9 and 10, SC191; 11 and 12, SC188. Odd-numbered lanes are unstimulated; even-numbered lanes are stimulated with IL-1. (*b*) Radioautography using cell line SC190. Lanes 1 and 2 are unstimulated. Lanes 3 and 4, 5 and 6, and 7 and 8 were stimulated with 0.1, 1.0 and 10.0 ng of IL-1/ml respectively. Lanes 9 and 10, 11 and 12, and 13 and 14 were stimulated with 0.1, 1.0 and 10.0 ng of TNFα/ml respectively. Odd-numbered lanes are [^{35}S]methionine-labelled; even-numbered lanes are [^{35}S]cysteine-labelled. Abbreviations: Coll, collagenase; TIMP, tissue inhibitor of metalloproteinases; IL-6, interleukin-6; β2m, β2-microglobulin; IL-8, interleukin-8; gro, 'gro' (growth-related) protein. The bands were identified by *N*-terminal sequencing.

RESULTS

While studying the synthesis of secreted proteins by connective tissue cells [4], we observed that one of the major proteins secreted by synovial cells was a 39 kDa protein (Fig. 1*a*, lanes 5–12). This protein was absent in the media obtained from lung (lanes 1 and 2) and skin (lanes 3 and 4) fibroblasts. The amount of the secreted protein was not altered by IL-1 (Fig. 1*b*, lanes

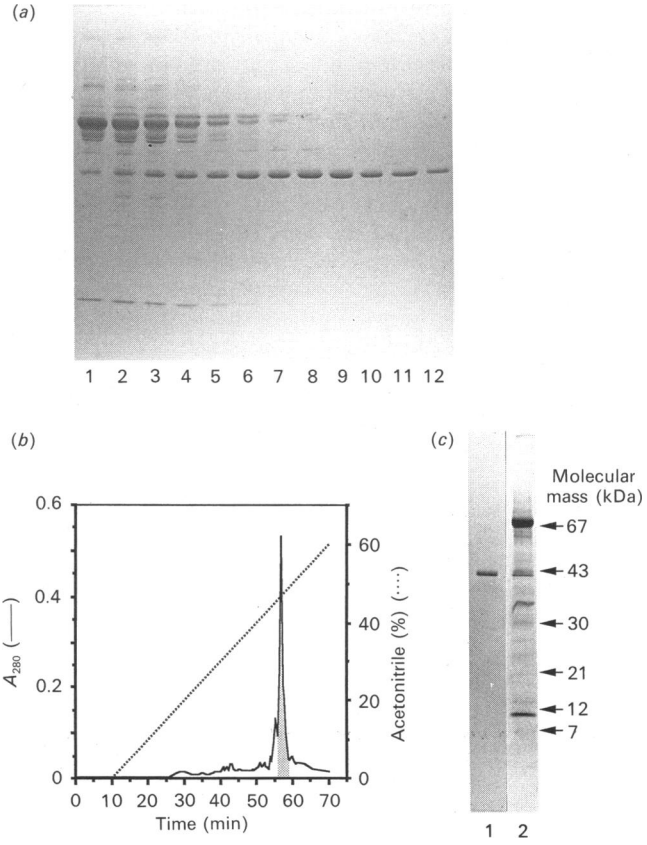

Fig. 2. Purification of synovial protein

(*a*) SDS/PAGE of heparin–Sepharose fractions. NaCl concentrations (M): lane 1, 0.24; 2, 0.36; 3, 0.41; 4, 0.45; 5, 0.48; 6, 0.53; 7, 0.57; 8, 0.60; 9, 0.70; 10, 0.80; 11, 0.88; 12, 0.91. (*b*) Chromatograph of reverse-phase h.p.l.c. fractions. (*c*) SDS/PAGE of reverse-phase h.p.l.c. fractions. Lane 1, fraction corresponding to the shaded area in (*b*); lane 2, starting material (Coomassie Brilliant Blue + silver stain).

3–8) or TNFα (lanes 9–14), although the expression of some other proteins was substantially altered by exposure of the synovial cell monolayer to IL-1 or TNF. The secretion was maintained through several passages. For example, in Fig. 1(*a*) the protein in lanes 7 and 8 is from the 7th passage, whereas that in lanes 11 and 12 is from the 22nd passage.

Besides the apparently normal skin and lung fibroblasts, we also studied skin fibroblasts from patients with various diseases (PSS, Fanconi, Cockayne and Bloom syndrome). None of these cell lines expressed detectable amounts of a 39 kDa protein (results not shown).

The 39 kDa band in the polyacrylamide gels could be stained with the periodic acid–Schiff reaction. This suggests that this protein is a glycoprotein. Occasionally in some cell lines a 39 kDa doublet could be detected rather than a single band. This may be due to differential glycosylation. In synovial fluid from rheumatoid arthritis patients we can also see a protein of similar relative mobility which can be stained by the periodic acid–Schiff reaction (results not shown).

Purification

Concentrated medium from synovial cell cultures was analysed on heparin–Sepharose affinity chromatography columns. The relative concentration of the 39 kDa protein in the fractions was determined by SDS/PAGE. The concentration of the 39 kDa material was found to be the highest in the fractions eluting at 0.6–0.7 M-NaCl (Fig. 2*a*). Fractions eluting between 0.5 and 0.9 M-NaCl were pooled and further purified on a reverse-phase C_{18} h.p.l.c. column (Millipore/Waters). The fractions corresponding to the absorbance peak at 46–48% acetonitrile concentrations (Fig. 2*b*) were collected and analysed by SDS/PAGE. Only a single band could be detected by silver staining (Fig. 2*c*).

(*a*)

Amino acid	Composition (%)
Asp + Asn	12.5
Thr	7.7
Ser	8.5
Glu + Gln	9.1
Pro	3.7
Gly	10.5
Ala	8.3
Cys	N.D.
Val	4.0
Met	1.7
Ile	4.6
Leu	9.4
Tyr	3.7
Phe	5.1
His	2.3
Lys	5.7
Arg	2.8
Trp	N.D.

(*b*)

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          1          11          21
Human     YKLVCYYTSWSQYREGDGSXFPDA...
               ^
Bovine    YKLIXYYTSWSQYREGDGSXFPDA...
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Fig. 3. Sequence comparison of human synovial protein and bovine mammary protein

(*a*) Amino acid composition of the synovial protein. N.D., not detected. (*b*) *N*-Terminal amino acid sequence of the human synovial protein, compared with that of the bovine mammary protein [10]. The caret (^) indicates the only non-identical amino acid residue found between the two proteins in the first 24 residues.

Amino acid composition and sequence analysis

Fractions eluting at 0.5–0.9 M-NaCl from the heparin–Sepharose column were pooled, concentrated and applied to an SDS/polyacrylamide gradient gel, blotted and submitted for amino acid analysis or sequencing (Fig. 3). The human sequence in Fig. 3(*b*) was searched against the NBRF [8] protein database using the FASTA [9] program, and against the Genbank database using the TFASTA program [9]. A bovine mammary protein was found in the protein databank which differed by only one amino acid from the synovial cell protein (Val-4 to Ile-4) in the *N*-terminal 24 amino acid segment [10]. No other similar proteins were found.

DISCUSSION

We have identified a 39 kDa protein secreted by all the synovial cell lines ($n = 10$) but not by any of the fibroblast cell lines ($n = 8$) examined. Based on the observation that this glycoprotein binds heparin, we developed a two-step purification scheme which made it possible to purify the 39 kDa protein to apparent homogeneity and to determine the *N*-terminal amino acid sequence. The sequence indicates that this protein is the human homologue of a bovine protein isolated from non-lactating cows [10]. Both proteins can be stained in the periodic acid–Schiff reaction and have identical mobilities on SDS/PAGE. The fifth residue in the bovine protein was hypothesized to be a cysteine residue in [10], because no other amino acid could be determined. We confirmed this residue to be a cysteine by radiosequencing in the human sequence. Assuming that the blank cycles in the bovine and the human sequences correspond to identical amino acids (possibly cysteine), there is only one amino acid difference between the two proteins within the first 24 residues, and even this substitution is a conservative one (Val to Ile). The 39 kDa protein appears to be a highly conserved protein which may imply an important functional role.

Though it may seem to be surprising that this protein is found in two such different organs as the mammary gland and the synovium, there are also similarities in their expression: (1) both proteins are secreted to the extracellular compartment, and (2) both proteins are secreted by a relatively quiescent tissue. The mammary protein is isolated during the involutionary and non-lactating phase, whereas the synovial cell protein is produced by a very slowly dividing connective tissue cell type.

It will be of interest to study the expression of this protein in other slowly dividing connective tissue cell types, e.g. in chondrocytes. The isolation and characterization of this protein may be useful by providing a marker distinguishing the synovial cell from fibroblasts.

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