Mapping of proteins from cultured fibroblasts of synovial and subcutaneous origin by high resolution two-dimensional polyacrylamide gel electrophoresis

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SUMMARY Nine different cell lines of human (A) rheumatoid and (B) normal synovial fibroblasts and of (C) normal skin fibroblasts were obtained from tissue explants and grown as monolayers. The cellular protein synthesis was studied by high resolution two-dimensional polyacrylamide gel electrophoresis. Between the fourth and 10th passage of the cells in culture apparently no consistent differences were found which could be ascribed to the origin of the cells, to the rheumatoid state of the synovial tissue, or to the increasing age of the cultured fibroblasts.

Key words: cells: cultured, tissue associated proteins, disease associated proteins, cell aging.

Cultured cells obtained from the innermost layer of the human synovial tissue, the synovial membrane, are widely used in studies on rheumatoid arthritis (RA). Primary cultures of these cells, designated synovial fibroblasts, have been established by different techniques.[1-6] Possible coculturing of fibroblasts of synovial and connective tissue origin is a particular problem when the 'explant' technique[1][2] or the 'adherent cell' technique[5][6] is used and thus emphasises the need for comparative studies aimed at characterising fibroblasts of different origin.

Enhanced synthesis of a variety of cellular proteins is reported to be a hallmark of the rheumatoid synovium,[7] but the data available from studies on cultured synovial fibroblasts until now are somewhat controversial. The technique of high resolution two-dimensional polyacrylamide gel electrophoresis (2D-PAGE),[8-10] based on separation by isoelectric focusing in the first dimension and sodium dodecyl sulphate (SDS) gel electrophoresis in the second, makes possible the recognition of more than 1000 different proteins (polypeptide chains) originating from applied cellular material (see e.g., Duncan and McConkey[11]). A recent 2D-PAGE study[12] on peripheral blood leucocytes from patients with active RA has suggested the occurrence of possible disease marker proteins.

In the present study the 2D-PAGE technique was used to map the cellular proteins from cultured rheumatoid and normal fibroblasts originating from the synovial membrane, as compared with those from subcutaneous fibroblasts. The purpose was to reveal possible differences which could be ascribed to either the tissue of origin, the disease, or to the passage numbers of the cultured cells.

Materials and methods

SYNOVIAL TISSUE AND CULTURE METHOD
Synovial tissue specimens were obtained during surgery from three adult patients with classical seropositive RA[13] who were admitted for synovectomy of the knee, elbow, or wrist joint. None of the patients received remission inducing antirheumatic drugs at the time of surgery. Light microscopic examinations showed chronic inflammation of the synovium. Synovial tissue from three non-rheumatoid patients admitted for knee surgery because of ruptured meniscus or chondromalacia patellae was also obtained.

The synovial membrane was dissected from the supporting connective tissue, cut into pieces of approximately 1 mm³, and explanted into Falcon tissue culture flasks (25 cm³), about 10 pieces per flask. The maximum time between surgery and plating was three hours. The explants were maintained in Dulbecco's modification of Eagle's medium supplemented with L-glutamine (0·6 g/l), 2·5% fetal calf serum, 15% horse serum (Flow Laboratories, UK), penicillin (10⁵ U/l), streptomycin (100 mg/l), and nystatin (5×10⁴ U/l) (Gibco Laboratories, NY, USA). They were incubated at 37°C in the presence of 5% carbon dioxide in air at pH 7·2–7·3. Sufficient primary outgrowth to permit subculturing was usually achieved 3–4 weeks after explantation. The cells were detached from the flasks by incubation with 0·05% trypsin 1:250 and 0·02% ethylene diamine tetra-acetic acid (EDTA) in salt solution (Flow Laboratories) for 5 min. After centrifugation the cells were resuspended in fresh medium and grown as monolayers in Costar tissue culture flasks (75 cm³). The morphology and behaviour of the cells were studied by phase contrast microscopy.

FIBROBLASTS FROM SUBCUTANEOUS TISSUE
Fibroblasts were obtained from biopsy specimens from the volar upper arm of three healthy persons. Primary cultures were established from tissue specimens explanted to Petri dishes (5 cm) and kept in Eagle's minimal essential medium (MEM) (Flow) supplemented with serum and antibiotics as described above for 10–14 days before subculturing. After the first passage the skin fibroblasts were grown as the synovial fibroblasts.

RADIOLABELLING OF CELLULAR PROTEINS
In order to avoid analytical variations the cells from the various passages (3, 5, 7, and 9) were kept frozen until use. The experiments were then performed simultaneously on all cell lines and passages. The cells were plated in duplicate in flat bottom multiwell plates (16 mm, Costar, MA, USA); 1·5×10⁴ cells/well, in 1 ml Eagle's MEM were used. After 24 h the medium was replaced with 500 μl of methionine deficient (1 g/l) medium containing 8–10×10⁴ μCi/l of ³⁵S-methionine of high specific activity (1420 kCi/mol, Amersham, Buckinghamshire, England). The cells were incubated for 48 h, rinsed in isotonic phosphate-buffered saline and those from the fourth, sixth, eighth, and 10th passages were harvested by addition of 50 μl/well of a solubilisation buffer containing urea (9 mol/l), 2-mercaptoethanol (50 ml/l), ampholines (2 ml/l) (LKB, Stockholm, Sweden), and a non-ionic detergent Nonidet P40 (NP-40) (40 ml/l) (Sigma Chemi-

cal Co., MO, USA). The lysed samples were centrifuged for 1 min at 9000 *g* in a Beckman Microfuge B (Beckman Instruments, CA, USA); insoluble material was sedimented. Aliquots (15 μl) of the supernatants were analysed by 2D-PAGE.

TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS
The equipment was purchased from Electro-Nucleonics, TN, USA, and based on the ISO-DALT system described by Anderson and Anderson.[9][10] Twenty samples were analysed simultaneously. An LKB 3371 power supply was used for isoelectric focusing and an HP 64483 (Hewlett-Packard, CA, USA) for the SDS electrophoresis. A refrigeration bath (model 2160, Forma Scientific, OH, USA) kept the temperature at 10°C during the second separation. The first dimension gels (4% acrylamide) for isoelectric focusing contained urea (9 mol/l), ampholines, pH 3·5–10 (50 ml/l), and NP-40 (30 ml/l). The second dimension slab gels were 8–18% linear gradient polyacrylamide gels containing 0·1% SDS. Internal isoelectric point standards produced by carbamylation of rabbit muscle creatine kinase were used in all gels.[14] After the 2D-PAGE separation gels were fixed and stained with Coomassie Brilliant Blue R250 (BioRad Laboratories, CA, USA) in order to visualise the non-radioactive internal standards. The gels were then dried (BioRad Slab Gel Drier, model 1125) and autoradiographed for 2–3 weeks on Kodak X-Omat AR film (Eastman Kodak, NY, USA). The autoradiograms were compared visually.

Results

MORPHOLOGY AND GROWTH CHARACTERISTICS OF CULTURED CELLS
The great majority of the synovial fibroblasts grown as monolayers were spindle-shaped and stellate cells, but a few multinuclear cells were also observed. The cells grew in a reticulate pattern. No conspicuous surplus of debris was noticed in the medium. The doubling time of the synovial fibroblasts was estimated to be about 2–3 days, whereas the skin fibroblasts proliferated more rapidly. The growth rate of the synovial fibroblasts decreased and they got an altered appearance at about the 10th passage. Spindle-shaped cells dominated the skin fibroblast cultures, and they grew with a more parallel orientation compared with the synovial fibroblasts.

CELLULAR PROTEINS IN SYNOVIAL AND SKIN FIBROBLASTS
2D-PAGE analysis of ³⁵S-methionine labelled cell

proteins showed no reproducible differences in the protein patterns of the various cell lines. This was also the case when proteins derived from normal and rheumatoid synovial cells were compared. Furthermore, an increasing age of the cultured cells apparently did not alter the protein maps. A few spots out of several hundred mapped proteins or peptides on each gel varied between the individual lines of each cell type, between the different cell types, or between the various passages of the individual cell lines. These changes, however, were not consistent and could not be ascribed to the tissue of origin, the health or disease of the donors, or to the passage numbers of the cell lines. Fig. 1 shows the mapped ^{35}S-labelled proteins from (A) rheumatoid and (B) normal synovial fibroblasts, and (C) skin fibroblasts. These patterns are representative for all cells analysed.
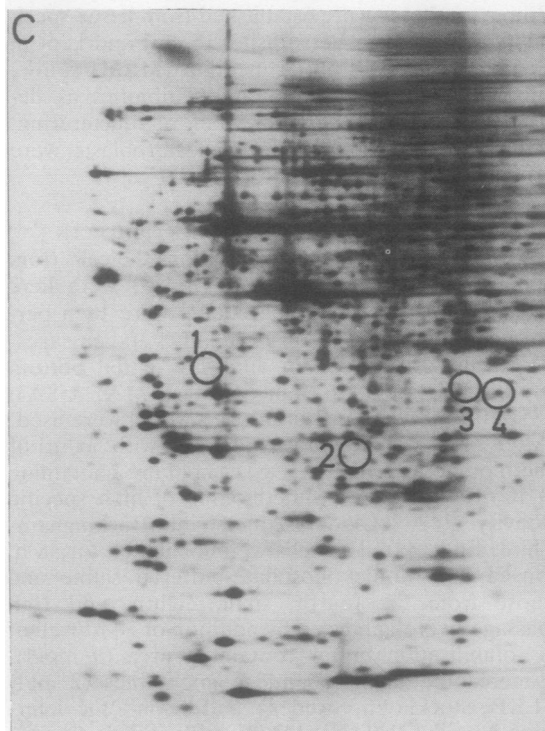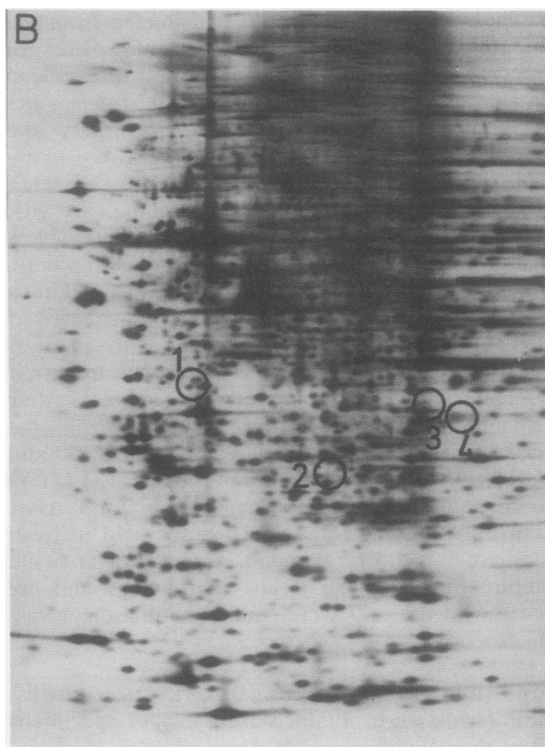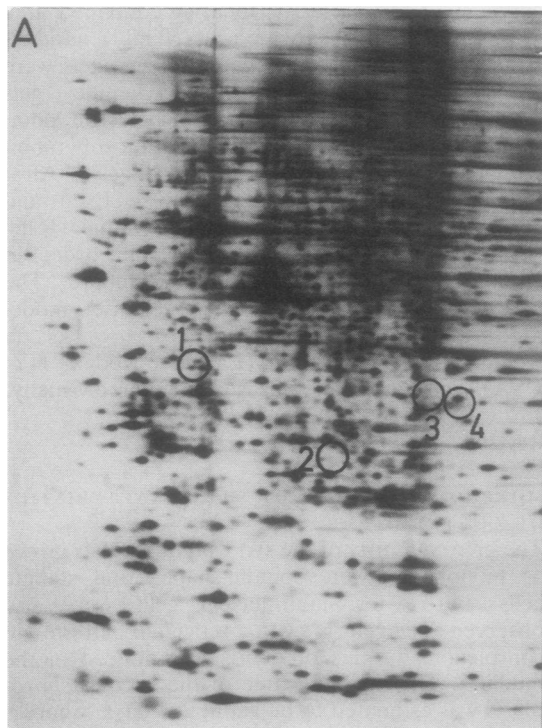

Fig. 1   *Two-dimensional protein patterns of cultured human (A) rheumatoid and (B) normal synovial fibroblasts and (C) skin fibroblasts, all from the fourth passage. The proteins were separated by two-dimensional polyacrylamide gel electrophoresis and visualised by autoradiography. The gels are oriented with the acidic end to the left, pH range 3·5–10. Molecular weight range in the vertical direction is from 200 000 (top) to 10 000 (bottom). The open circles 1–4 exemplify the non-consistent variations in protein patterns found in a few protein spots out of several hundred mapped proteins.*

Discussion

The cultured synovial fibroblasts in the present study share a number of morphological and growth characteristics with those cells usually regarded as synovial cells or fibroblasts, after the 'explant' technique has been applied to establish primary cell cultures.[15-19]

The lack of differences in the 2D-PAGE protein maps of cultured fibroblasts of different origin is in agreement with the early studies of Castor *et al.*[15] who used one-dimensional starch gel electrophoresis. Their comparison of 13 primary fibroblast cell lines derived from various anatomical sites (human synovial membrane, skin, periosteum, pleura, pericardium, and peritoneum) showed no reliable differences in protein patterns.

Parrott *et al.*[20] found no difference in the ratio of collagen type I and III in normal and rheumatoid synovial fibroblasts during the first two passages in culture. On the other hand in primary organ cultures the rheumatoid synovial tissue produced relatively more of type III collagen than normal tissue and isolated rheumatoid fibroblasts in cell culture. McGuire *et al.*[21] also found that the enhanced synthesis of collagen in cultured rheumatoid synovial (adherent) cells did not persist beyond the first day in culture. This might indicate that the alterations in collagen synthesis in rheumatoid synovial tissue may be partly dependent on local mediators of inflammation or cell interactions present in organ cultures but absent in cell cultures. This might also be one explanation of the numerous reports on enhanced protein synthesis in rheumatoid synovial tissue in vivo (for review, see ref. 7). Consequently one advantage of examining synovial fibroblasts beyond the first two to three passages is that one is dealing with cultures less contaminated with other cells from the synovial tissue. In view of the data discussed above it is perhaps not surprising that there were no consistent differences in the 2D-PAGE protein maps of our various cell lines.

It is well reported that human fibroblasts have a limited replicative life span in primary cultures,[22] and that the aging of cells in vitro is associated with alterations in the cellular proteins.[23] The possibility therefore also exists that some of the specific cell characteristics initially present in rheumatoid synovial fibroblasts might be lost in culture within the first few passages of the cells. Because of the low cell gain achieved with the 'explant' technique, experimental use of the first fibroblast passages in our longitudinal study was not possible. The lack of age-associated changes in 2D-PAGE protein patterns between the fourth and 10th passage of the cell lines leads us to suggest that experiments applying long-term cultures of synovial fibroblasts may be carried out up to the 10th passage of the cells.

In conclusion the highly sensitive and efficient separation method used in this study, 2D-PAGE (see, e.g., ref. 24), revealed no consistent differences in the protein maps of fibroblasts from different tissues of origin, between synovial fibroblasts obtained from RA patients and healthy persons, or between various passages of cells in culture. The lack of differences may be due to mediators/factors lost in cell culture and to early aging effects. It does not, however, preclude the possibility that the proteins synthesised in vivo may differ, and that the cultured fibroblasts are indeed derived from tissues with different biological functions.

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