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Xenografts of Articular Chondrocytes in the Nude Mouse

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Summary: Subcutaneous transplantation of articular chondrocytes isolated enzymatically from immature rabbits and dogs into athymic (*nu/nu*) mice resulted in the formation of hyaline cartilaginous nodules. Graft rejection was seen when the cells were injected into heterozygous (*nu/+*) mice. Radiosulfate-labeled proteoglycan extracted from the xenografts had a high buoyant density and was digested by chondroitinase ABC. A monomeric preparation of proteoglycan (A1-D1) contained a small quantity of aggregate as assessed by gel chromatography and gel electrophoresis. Almost no incorporation of ^{3}H-thymidine was found by autoradiography. The matrix did not become calcified over the course of 42 days. The nude mouse system lends itself to testing a variety of problems in the biology of cartilage. These include the redifferentiation of chondrocytes following dedifferentiation in vitro. Species differences were found in this regard. The nodules formed by rabbit articular chondrocytes, grown in monolayer culture for up to 14 days, had a hyaline chondroid character. Dog chondrocytes that had "dedifferentiated," during 14 days of culture prior to transplantation, formed a graft that had a sparse fibrous rather than hyaline matrix.

Key words: Articular chondrocytes — Nude mouse — Xenograft — Dedifferentiation.

Experimental grafts of cartilage and its cells have been investigated extensively for potential therapeutic applications and for tracing the sequences of endochondral ossification [1–4]. Whole cartilage is uniquely resistant to immunological attack, probably because its matrix hinders access of antibodies sterically [2, 5–7]. There is considerable evidence that chondrocytes proper possess histocompatibility antigens that ultimately embarrass survival of homografts and xenografts [2, 8, 9]. Several immunologically privileged systems for studying these phenomena have been employed at times: the anterior chamber of the eye [10]; syngeneic hosts [6]; and the hamster cheek pouch [4, 11]. To these we now add the athymic nude (*nu/nu*) mouse. This system lends itself to investigating the preceding and additional aspects of the biology of chondrocytes.

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Materials and Methods

Experimental Design

Cartilaginous nodules were produced by subcutaneous inoculation of freshly dissociated articular chondrocytes from rabbits and dogs into nude mice. The following experiments were designed to evaluate several characteristics of the cartilage formed: validation, through karyotyping, that the nodules are true xenografts rather than foci of metaplasia of host mesenchyme; histochemical and biochemical documentation of the chondroid nature of the matrix formed; autoradiographic estimation of the degree of chondrocytic proliferation; and a determination whether "dedifferentiation" of chondrocytes in monolayer culture can be reversed when the cells are restored to a physiological environment.

Reagents

Dulbecco–Vogt Medium (DMEM), colcemid, Gey's balanced salt solution (GBSS), penicillin–streptomycin solution 10,000 U/ml and 10,000 μg/ml, respectively (P/S), fetal bovine serum (FBS), and sterile trypsin solution were purchased from GIBCO, Grand Island, NY; ^{3}H-thymidine, specific activity 3 Ci/mmol (^{3}H-dThd) from Schwartz–Mann, Orangeburg, NY; $Na^{35}SO_4$ (99% radionuclidic purity, specific activity 500–900 mCi/mmol) from New England Nuclear, Boston, MA; testicular hyaluronidase, lyophilized trypsin and clostridial collagenase (CLS-II), Worthington Biochemical Corp., Freehold, NJ; Nuclear track emulsion (NTB-2) from Kodak, Rochester, NY;

cetylpyridinium chloride (CPC), and trypan-blue stain, Sigma Chemical Co., St. Louis, MO; guanidinium chloride (GuCl), sequanol grade, Pierce Chemical Co., Rockford, IL; CsCl (density gradient-grade), Gallard Schlesinger, Carle Place, NY; Biogel A50m, Bio Rad, Richmond, CA; sodium acetate (NaAc), Fisher Scientific, Fairlawn NJ; and Monofluor, National Diagnostics, Somerville, NJ.

Animals

Twenty-four New Zealand White Rabbits (2–3 kg) and two young mongrel dogs less than one year old were used for donor cartilage. Eighteen 28- to 30-day-old homozygous (*nu/nu*) and two heterozygous (*nu/+*) nude mice were obtained from Charles River Breeding Laboratories (Wilmington, MA). They were housed as described by Gullino et al. [12].

Isolation and Culture of Chondrocytes

Cartilage was isolated from the shoulder, knee, and hip joints of the rabbits and from the shoulders of the dogs. Chondrocytes were dissociated from their matrix by sequential digestion with hyaluronidase, trypsin, and collagenase as previously described [13, 14].

Rabbit articular chondrocytes were cultured in monolayer flasks for 1, 2, 3, 4, 7, and 14 days [13, 14]. The inoculation density for days 1–4 was 6×10^5 cells/T75 flask; for days 7–14, 3×10^5. F12 medium supplemented with 10% FBS and 0.1% P/S was employed for the first week of culture. Thereafter, F12 was replaced by DMEM. The cells were detached with trypsin and washed three times with GBSS prior to transplantation. A single experiment was carried out with dog chondrocytes, cultured 14 days with DMEM. The grafts were examined histologically seven days after transplantation.

Transplantation and Graft Recovery

Viability was determined by trypan-blue (0.2% in GBSS w/v) exclusion. Freshly isolated cells were washed three times with GBSS. They were then suspended at $4–6 \times 10^6$ viable cells in 0.1 ml GBSS and injected subcutaneously into the dorsum by using a 26-gauge needle. Chondrocytes that had been cultured in vitro were injected at the same inoculum size.

The mice were killed with CO_2. Grafts were isolated under aseptic conditions and placed either in 10% neutral buffered formalin containing 1% CPC for histological examination, GBSS for cell culture, 4 *M* GuCl for proteoglycan analysis. Grafts for ^{3}H-dThd autoradiography were harvested three and seven days following transplantation. Control histological studies for graft rejection were carried out using (nu/+) heterozygous nude mice seven and 14 days after transplantation of rabbit articular chondrocytes.

Histology

Grafts were embedded in paraffin and cut at a thickness of 4–5 μm. Sections were stained wtih hematoxylin-eosin (H&E), and in selected instances with safranin O-fast green, Feulgen, Gomori reticulin, Verhoeff's elastin, periodic acid–Schiff (PAS), Von Kossa or alizarin red [15].

Autoradiographs

On days 3 and 7 after inoculation of rabbit chondrocytes, mice were labeled intraperitoneally for 4 h with 1 μ Ci ^{3}H-dThd in GBSS/g body weight. The grafts were fixed and sectioned as above. Slides were dipped in NTB-2 emulsion (1:1 distilled water), allowed to develop for 14 days, and counterstained with H&E. Small intestine and skin were taken as control tissues.

Karyotyping

After a portion of the xenograft was fixed for histology, the remainder was digested, cultured, and karyotyped as described elsewhere [16].

Proteoglycan Analysis

One mouse was injected at two sites with a total of 1.2×10^7 rabbit chondrocytes. Seven days later it was labeled intraperitoneally with 2 μ Ci $^{35}SO_4$/g body weight for 4 h. The grafts were then excised and placed immediately into 4 *M* GuCl. Proteoglycans were extracted from the diced nodules with 4 *M* GuCl, pH 5.8 [17], and dialyzed at 4°C for 18 h against 7 vol 0.05 *M* NaAc, pH 5.8. CsCl and 0.05 *M* NaAc pH were added to the retentate to yield a solution of density 1.5 g/ml and vol 4.0 ml. This solution was centrifuged at 90,000 *g* for 72 h in a Sorvall OTD65 ultracentrifuge. The tubes were then frozen in liquid N_2 and the bottom two-fifths by volume sawed off, filtered, and dialyzed against 0.05 *M* NaAc, pH 5.8. This fraction was referred to as the A1 fraction ("A" for associative conditions, i.e. 0.5 *M* GuCl; "1" refers to the most dense fraction in the gradient. This informal, but internationally accepted, nomenclature is described in detail in the review by McDevitt [18]). Over 95% of the ^{35}S counts were located in the A1 fraction, a finding that is consistent with the well-documented observation that proteoglycans have buoyant densities in excess of 1.7 g/ml. CsCl and GuCl were added to the A1 fraction to yield a solution of density 1.5 g/ml and a guanidinium concentration of 4 *M*. This again was centrifuged under the conditions employed for the associative gradient. The bottom two-ninths of the gradient was isolated and referred to as the A1-D1 fraction (i.e. the starting material was an A1 fraction, and 4 ml GuCl employed in the gradient dissociates proteoglycan aggregates and the conditions are therefore referred to as dissociative of "D", and the first, most dense fraction of the gradient was recovered). The A1-D1 fraction was dialyzed exhaustively against 0.5 *M* NaAc, pH 5.8. An aliquot of the proteoglycan fraction was chromatographed on Biogel A50m with 0.5 *M* NaAc, pH 6.8, as the eluting solution. Fractions were mixed with Monofluor and radioactivity monitored in a Searl Mark III scintillation counter. Proteoglycan fractions were also analyzed by composite, large-pore polyacrylamide (1.2%)–agarose (0.6%) electrophoresis [19] before and after digestion with chondroitinase ABC by the method of Oike et al. [20]. Chondroitin-4 sulfate (C4S), a gift of Dr. M. Mathews, University of Chicago, was employed as a standard in the chondroitinase digestions and subsequent electrophoretic analyses. The electrophoretic gels were stained with Stains All [21].

Results

The transplanted rabbit chondrocytes formed a discrete nodule, 5–10 mm in greatest dimension, by

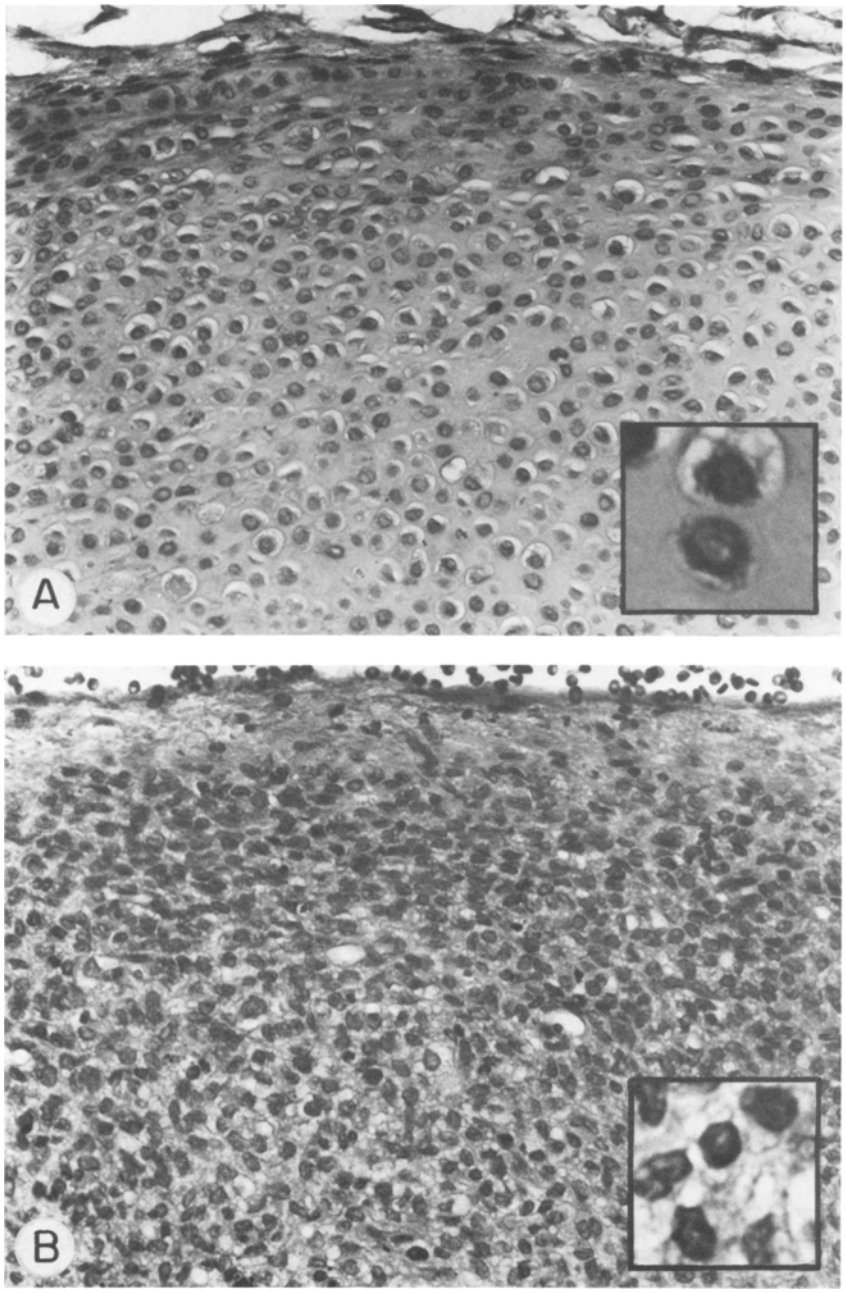

Fig. 1. Chondrocyte xenografts in nude mice, 7 days. **(A)** Rabbit chondrocytes, freshly isolated before grafting, are surrounded by a well-defined hyaline cartilaginous matrix. A delicate fibrous capsule at the upper margin merges imperceptibly with the chondroid tissue. **(B)** Dog chondrocytes, cultured 14 days in vitro prior to transplantation, are separated by scanty fibrillar intercellular material. H&E, ×200; insert, ×650.

day 7. There was no further increase in size during the six-week observation period. Histologically the nodules had a hyaline cartilaginous matrix that was stained intensely orange by safranin O. The nodules merged imperceptibly with an encapsulating delicate layer of fibroblastic cells (Fig. 1A). There was no inflammatory reaction except for an isolated miliary infiltrate of lymphocytes near the edge of a 28-day-old nodule. No reticulin, elastin, or calcific material was demonstrable in the matrix over the course of six weeks. The nuclei appeared viable throughout in the Feulgen preparations. Glycogen (diastase-soluble PAS) was present in the cytoplasm of many chondrocytes. Grafts of freshly isolated dog chondrocytes closely resembled those of rabbit cells.

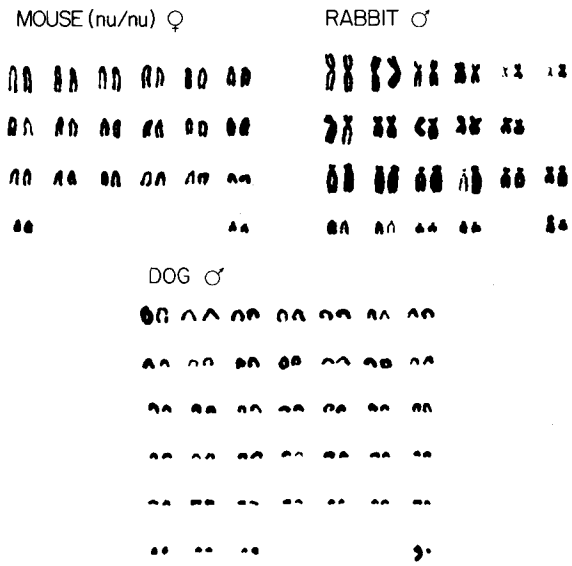

Fig. 2. Karyotype of nude mouse skin fibroblasts compared with rabbit and dog xenografts. The chromosome numbers and morphology are characteristic of each species. Compare Hsu and Benirschke [32].

Articular chondrocytes injected into the heterozygous nude mice showed early rejection of the chondroid nodules at day 7 in the form of heavy infiltration of the adjacent adipose tissue by mononuclear cells. By 14 days, extensive necrosis of cartilage accompanied by large numbers of polymorphonuclear leukocytes as well as mononuclear cells was present. Sections of the knee joints of these heterozygous mice disclosed no arthritic reaction.

Each of the nodules formed by the transplanted rabbit chondrocytes cultured for 1–14 days contained a copious safraninophilic matrix. Foci of necrosis were seen in the center of some nodules in which a large number of ($>10^7$) cultured chondrocytes had been injected. The presumption is that, in these instances, the cell mass had exceeded its blood supply. By contrast, dog chondrocytes that had been cultured for 14 days prior to transplantation formed less clearly defined nodules grossly. Thin-walled blood vessels were closely attached to and infiltrated the nodules. Histologically, the cultured dog chondrocyte grafts contained little extracellular material (Fig. 1B). The matrix was fibrillar and could not be stained by safranin O. Reticulin and elastin stains of the extracellular material were also negative.

That the nodules represented authentic grafts rather than cartilaginous metaplasia of host mesenchyme was shown by the karyotypes, characteristic of each species studied (Fig. 2). Autoradiographs failed to demonstrate incorporation of ^{3}H-dThd except by rare capsular cells at either three or seven days following the transplantation. Vigorous DNA synthesis was shown by the control tissues. The cells thus apparently did not divide, but survived and laid down copious cartilaginous matrix.

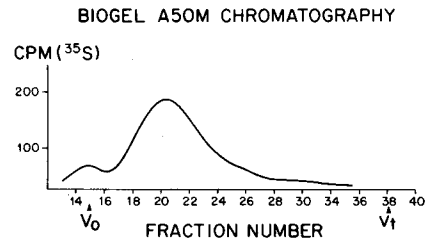

Fig. 3. Biogel A50m chromatography of A1-D1 fraction isolated from radiosulfate-labeled graft of rabbit chondrocytes in *nu/nu* mouse.

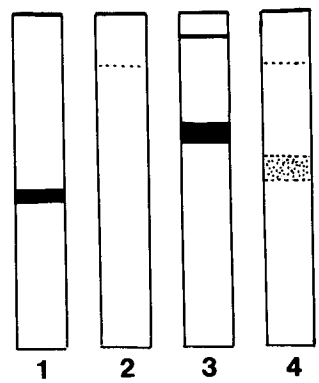

Fig. 4. Polyacrylamide–agarose electrophoresis of C4S and A1-D1 fraction from same nodule as Figure 3. (*Left to right*) gel 1: C4S; gel 2: C4S after digestion with chondroitinase ABC; gel 3: A1-D1 fraction from *nu/nu* mouse; gel 4: A1-D1 fraction after digestion with chondroitinase ABC. Heavily and lightly stained bands are depicted as black and speckled areas, respectively. The faintly stained, low-mobility band in the chondroitinase-digested specimens is due to the enzyme.

The radiosulfate-labeled proteoglycan isolated from the nodules had high bouyant densities in associative and dissociative CsCl density gradients. It eluted from the Biogel A50m column as a molecular species with a large hydrodynamic size (Fig. 3). The small proportion of radioactive material that eluted at the V_0 of the column probably represented contaminating proteoglycan aggregate.

Chondroitin 4-sulfate migrated on polyacrylamide–agarose gels as a single sharp band (Fig. 4). The D1 fraction migrated as a faintly staining peak with low mobility and a broad densely staining band that was slightly slower than the C4S band. Analogy with previous work [19] indicates that these bands represent aggregated and monomeric proteoglycans, respectively. Prior digestion with chondroitinase ABC completely abolished the C4S band. It reduced the staining of the major band of the D1 fraction considerably and increased its mobility.

Discussion

Previous investigators have employed histochemical and ultrastructural techniques to document the nature of the cartilage matrix formed when chondrocytes are injected into compatible hosts [6, 22, 23]. The present studies add several further types of information. Aside from describing features of the xenografts in a new host, the nude mouse, they provide data about proliferation of the chondrocytes when grafted. Under these conditions, the cells divide little or not at all. In addition, the composition of the matrix formed has been partially characterized chemically. The high buoyant density, gel chromatographic behavior, and sensitivity to chondroitinase ABC confirm that the material isolated as a D1 fraction is a chondroitin sulfate proteoglycan. The data do not warrant a categorical assertion that the material is typical of cartilage, but the small amount of aggregate demonstrable by gel chromatography and gel electrophoresis suggests tentatively that it is. Moskalewski [24] has shown that chondrocytes from the elastic cartilage of the ear of very young rabbits synthesize elastin when injected intramuscularly into closely related rabbits, but not when the cells are obtained from adults. Elastin was not seen histologically in the grafts of our articular chondrocytes from rabbits 2–3 months old.

Reference was made, in the introduction, to reports on the role of the matrix in differences observed between the behavior of grafts of cartilage and that of chondrocytes. Although acceptance of whole cartilage grafts has often been ascribed to low immunogenicity of the matrix, recent studies have demonstrated that both the collagen [25] and noncollagenous proteins—proteoglycan core and glycoprotein link—[26] are antigenic. These observations lend weight to the steric role of the matrix in the protection of the tissue. Chondrocytes injected into heterozygous nude mice formed a matrix rapidly but this did not prevent graft rejection. Thyberg and Moskalewski [4] have recently reviewed controversial literature on osteogenesis induced by transplantation of cartilage and chondrocytes. They found that rat epiphyseal chondrocytes, grafted into the hamster cheek pouch, became mineralized in three weeks. By contrast, the same cells injected into rat muscle gave rise not only to calcification but also subsequent bone formation. No calcification was found in the xenografts of rabbit chondrocytes in nude mice during the 42-day period observed.

The role of the microenvironment in governing chondroid expression and proliferation of articular and nonarticular chondrocytes in vitro continues to be studied actively. These cells divide vigorously in monolayer culture, but "dedifferentiate" with respect to the type of collagen and GAG profile they synthesize. The cartilaginous phenotypes are better preserved in suspension cultures (reviewed by Sokoloff [27]). Reexpression of Type II collagen and cartilage-like GAGs has been reported repeatedly when monolayer-cultured chondrocytes of rabbits are returned to a spinner environment or agarose gel [28–31]. The nude mouse graft system should a priori provide an even more physiological milieu than suspension culture for evaluating this phenomenon. In the present study, rabbit chondrocytes that had been cultured up to 14 days in monolayer flasks gave raise to nodules that contained abundant intercellular matrix. The latter has the histological and histochemical properties of cartilage glycosaminoglycans. Dog chondrocytes did not. This is one more apparent species difference in the in vitro behavior of mammalian chondrocytes [27]. Green [1, 14] was unable to find a residual graft in rabbit muscle that had been injected with cultured rabbit chondrocytes unlike muscle that had received freshly dissociated cells. The collagens synthesized in the nude mouse xenografts are now being investigated. The system thus proves suited to evaluating factors that govern reexpression of chondroid genes.

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References

1. Green WT Jr (1977) Articular cartilage repair. Behavior of rabbit chondrocytes during tissue culture and subsequent allografting. Clin Orthop 124:237–250.
2. Elves MW (1978) The immunobiology of joints. In: Sokoloff L (ed) The joints and synovial fluid, vol 1. Academic Press, New York, pp 331–406
3. Bentley G, Smith AU, Mukerjhee R (1978) Isolated epiphyseal chondrocyte allografts into joint surfaces. An experimental study in rabbits. Ann Rheum Dis 37:449–458
4. Thyberg J, Moskalewski S (1979) Bone formation in cartilage produced by transplanted epiphyseal chondrocytes. Cell Tissue Res 204:77–94
5. Loeb L (1926) Autotransplantation and homoiotransplantation of cartilage in the guinea-pig. Am J Pathol 2:111–122
6. Heyner S (1969) The significance of intercellular matrix in the survival of cartilage allografts. Transplantation 8:666–677
7. London WT, Fucillo DA, Anderson B, Sever JL (1970) Concentration of rubella virus in chondrocytes of congenitally infected rabbits. Nature 226:172–173

8. Malseed ZM, Heyner S (1976) Antigenic profile of the rat chondrocytes. Arthritis Rheum 19:223–231
9. Gertzbein SD, Tait JH, Devlin S, Rogargues S (1977) The antigenicity of chondrocytes. Immunology 33:141–145
10. Urist MR, Adam T (1968) Cartilage or bone induction by articular cartilage. Observations with radioisotope labelling techniques. J Bone Joint Surg 50B:198–215
11. Kaminski J, Kaminski G, Moskalewski S (1980) Species differences in the ability of isolated eipiphyseal chondrocytes to hypertrophy after transplantation into the wall of the syrian hamster cheek pouch. Folia Biol (Krakow) 28:27–38
12. Gullino PM, Ediger RD, Giovanella B, Merchant B, Outzen HC, Reed ND, Wortis HH (1976) Guide for the care and use of the nude (thymus-deficient) mouse in biomedical research. ILAR News 19 (2):M1–M20
13. Sokoloff L, Malemud CJ, Green WT Jr (1970) Sulfate incorporation by articular chondrocytes in monolayer culture. Arthritis Rheum 13:118–124
14. Green WT Jr (1971) Behavior of articular chondrocytes in cell culture. Clin Orthop 75:248–260
15. Lillie RD (1965) Histopathologic technique and practical histochemistry, 3rd edn. McGraw-Hill, New York
16. Krystal G, Morris GM, Lipman JM, Sokoloff L (1983) DNA repair by articular chondrocytes. I. Unscheduled DNA synthesis following ultraviolet irradiation in monolayer culture. Mech Ageing Dev 21:83–96.
17. Prins APA, Lipman JM, McDevitt CA, Sokoloff L (1982) The effect of purified growth factors on rabbit articular chondrocytes in monolayer culture. 2. Sulfated proteoglycans. Arthritis Rheum 16:1228–1238
18. McDevitt CA (1981) The proteoglycans of hyaline cartilage and the intervertebral disc in ageing and osteoarthritis. In: Glynn LE (ed) Handbook of inflammation, vol 3: Tissue repair and remodeling. Elsevier–Holland, Amsterdam, pp 111–143
19. McDevitt CA, Muir H (1971) Gel-electrophoresis of proteoglycans and glycosaminoglycans on large pore composite polyacrylamide–agarose gels. Anal Biochem 44:612–622
20. Oike YO, Kimata K, Shinomura T, Nakazawa K, Suzuki S (1980) Structural analysis of chick embryo cartilage proteoglycan by selective degradation with chondroitin lyases (chondroitinases) and endo-β-D-galactosidase (keratanase). Biochem J 191:193–207
21. Dahlberg A, Dingman CW, Peacock AC (1969) Electrophoretic characterization of bacterial polyribosomes in agarose–acrylamide gels. J Mol Biol 41:139–147
22. Moskalewski S, Kawiak J (1965) Cartilage formation after homotransplantation of isolated chondrocytes. Transplantation 3:737–747
23. Chesterman PV, Smith AU (1968) Homotransplantation of articular cartilage and isolated chondrocytes. J Bone Joint Surg 50B:184–215
24. Moskalewski S (1981) The elastogenetic process in transplants and cultures of isolated auricular chondrocytes. Connect Tissue Res 8:171–181
25. Gay S (1983) Immunology of collagen. In: Wagner BM, Fleischmajer RF (eds) Connective tissue and diseases of connective tissue. IAP Monograph. Williams and Wilkins, Baltimore pp 120–128
26. Poole AR, Pidoux I, Reiner A, Tangl H, Choi H, Rosenberg L (1980) Localization of proteoglycan monomer and link protein in the matrix of bovine articular cartilage: an immunohistochemical study. J Histochem Cytochem 28:621–635
27. Sokoloff L (1980) In vitro culture of joints and articular tissues. In: Sokoloff L (ed) The joints and synovial fluid, vol 2. Academic Press, New York, pp 1–26
28. Srivastava VML, Malemud CJ, Sokoloff L (1974) Chondroid expression by lapine articular chondrocytes in spinner culture following monolayer growth. Connect Tissue Res 2:127–136
29. Deshmukh K, Kline WG (1976) Characterization of collagen and its precursors synthesized by rabbit articular cartilage cells in various culture systems. Eur J Biochem 69:117–123
30. Norby DP, Malemud CJ, Sokoloff L (1977) Differences in the collagen types synthesized by lapine articular chondrocytes in spinner and monolayer culture. Arthritis Rheum 20:709–716
31. Benya PD, Shaffer JD (1982) Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 30:215–224
32. Hsu TC, Benirschke K (1967) The atlas of mammalian chromosomes, vol 1. Springer-Verlag, New York