J. Tiss. Cult. Meth. 15:139–146, 1993

0271-8057/93 $01.50+0.00

THE STUDY OF REPRESENTATIVE POPULATIONS OF NATIVE AGGRECAN AGGREGATES SYNTHESIZED BY HUMAN CHONDROCYTES IN VITRO

M. Cornelissen, G. Verbruggen[1], A. M. Malfait, E. M. Veys, C. Broddelez, and L. De Ridder

Histology Laboratory, University of Ghent, Belgium (M.C., L.DR.); and Department of Rheumatology, Ghent University Hospital, De Pintelaan 185, B-9000, Ghent, Belgium (G.V., A.M., E.V., C.B.)

SUMMARY: Chondrocytes were cultured in gelified agarose. Aggrecans, accumulated during culture, were liberated by agarase digestion of the artificial agarose matrix and studied by electron microscopy. The culture system and the specific method used to liberate the extracellular macromolecules enabled us to study the whole population of de novo synthesized, native aggrecan aggregates. The average length of the aggrecans was 188 nm. Aggrecans were observed as free molecules or attached on hyaluronan chains. In these aggregates no free binding sites on the hyaluronan chain were observed, and the average distance between the aggrecans was 27 nm. Consequently, the length of the hyaluronan chain defines the molecular size of the aggregate. Mature human articular cartilage cells were found to synthesize a relatively small proportion of very large aggrecan aggregates with over 100 aggrecans attached to a single hyaluronan chain. However, the average aggrecan aggregate carried about 12 aggrecans.

Key words: aggrecans; electron microscopy; articular cartilage; chondrocytes; in vitro.

I. INTRODUCTION

Because cartilage aggrecan aggregates resist extraction with associative solvents, electron microscopic studies of aggrecans are predominantly performed with aggregates obtained by dissociative extraction. After dissociation of the matrix molecules in situ, the different parts of the molecules reassociate under appropriate associative conditions to form aggrecan aggregates. The characteristics of these reconstituted aggregates have been studied extensively, for mature as well as for immature cartilage (Buckwalter and Rosenberg, 1982; Buckwalter et al., 1984; Buckwalter et al., 1985; Front et al., 1989; Kimura et al., 1978; Rosenberg et al., 1970; Rosenberg et al., 1975; Thyberg et al., 1975).

However, it cannot be taken for granted that the different constituents of the aggregate, e.g., the hyaluronan backbone, the aggrecan, and the link-glycoproteins, are as effectively extracted from the tissue. Furthermore, inasmuch as the extracted materials are reassociated at random into new aggrecan aggregates, these new aggregates are not per se identical to the original ones.

For these reasons, attempts were made to extract native aggrecans from tissues using non-dissociative (associative) solvents. However, electron microscopic studies on native aggrecan aggregates obtained by associative extraction are less extensive. The disadvantage of this extraction procedure is the low yield of aggrecans. Only about 20% of the tissue aggrecans could be liberated from mature cartilage (Brandt and Muir, 1969) or from cultured embryonic chicken limb bud cartilage (Kimura et al., 1978). Using highly hydrated chondrosarcoma tissue, about 60% of the tissue aggrecans were extracted (Faltz et al., 1972). In view of these findings it is still of value to develop methods for the study of the native, non-reconstituted aggrecan aggregate. The aim of the present work was to describe and evaluate a method for obtaining a large and representative portion of non-dissociated native aggrecan aggregates, synthesized by phenotypically stable, mature human articular cartilage chondrocytes in vitro.

II. MATERIALS

A. Equipment

1. Laminar air flow, Gelaire, class 100, Gelman Instruments[1]
2. Centrifuge, table model, Econo Spin, Sorvall Instruments[2]
3. Modular incubator chamber, box 977, Billups-Rothenberg[3]
4. pH meter, model 7,[2]
5. Micro carrier magnetic stirrer, Bellco Glass,[4]
6. Balance, H10W, Mettler[5]
7. Electron microscope, 1200 EX II, Jeol[6]
8. Vacuum evaporator, JEE 4B[6]
9. Copper grids, G200, Laborimpex[7]
10. Platinum-palladium wire, 0.2 mm, E 412-1[7]
11. Transferpettor, type Digital, positive displacement micropipette (10 μl), Brand[1]
12. Ubbelohde viscosimeter, no. 2450101, Schott[8]

[1] To whom correspondence should be addressed.

B. Plastics and glassware
   1. Spinner flasks, 100 ml, no. 1967-00100[4]
   2. Sterile Falcon polystyren round-bottom tube with cap (17 × 100 mm), no. 22310109, Becton Dickinson[9]
   3. Pasteur pipettes, no. 1504250[1]
   4. Nunc cryotubes, 3.6 ml, 366524 N 1087-1A, Nunc[10]
   5. Sterivex-GS, 0.22-μm pore, SVGS 010 15, Millipore[11]
   6. Transferpettor-Caps (10 μl), no. 701902,[1]
   7. Glass slides, no. 1169463[1]
   8. Eppendorf tubes, no. 3212, Costar[12]
   9. Petri dish (100 mm), no. 1154142[1]

C. Chemicals and culture media
   1. Dulbecco's modified Eagle's medium (DMEM) (with L-glutamine, 1000 mg/liter D-glucose, sodium pyruvate, and 25 m*M* HEPES), no. 041-02320, GIBCO[13]
   2. DMEM powdered medium, (with L-glutamine, 4500 mg/liter D-glucose, without sodium pyruvate and sodium bicarbonate), no. 074-02100[13]
   3. Sodium bicarbonate (tissue culture grade), no. 066-01810[13]
   4. Fetal bovine serum (FBS), no. 013-06290[13]
   5. Antibiotic/antimycotic solution (100×) (10 000 U penicillin, 10 000 μg streptomycin, 25 μg Fungizone per ml), no. 043-05240[13]
   6. L-Glutamine, no. 066-01051[13]
   7. Hyaluronidase, type V sheep, no. H-3506, Sigma[14]
   8. Pronase E from *Streptomyces griseus*, no. 7433.000120[14]
   9. Collagenase, type 1A from *Clostridium histolyticum*, no. C-9891[14]
   10. Ultra-low gelling temperature agarose (type IX), no. A-5030[14]
   11. L(+)ascorbic acid sodium salt, no. 11140, Fluka[15]
   12. Agarase 3-glycanohydrolase from *Pseudomonas atlantica*, no. A-6306[16]
   13. EDTA di sodium salt, no. 1412, UCB[1]
   14. 6-Aminocaproic acid, no. K2692818, Merck[16]
   15. Benzamidiniumchloride, no. 820122[16]
   16. Alfa-toluene-sulfonyl chloride, 98%, 17.049.74, Janssen Chimica[17]
   17. Phosphate buffered saline (PBS), 042-04200[13]
   18. Hyaluronan, Hyalectin^R, no. 24117, Fidia[18]
   19. Tris-(hydroxymethyl)-aminoethane, no. 274676188[15]
   20. Titriplex III (EDTA), no. K2692818[16]
   21. Hydrochloric acid (HCL), 1 mol/liter, no. 9757.1000[16]
   22. Ammonium acetate (CH3COONH4), no. 1116.0500[16]
   23. Cytochrome C, type VI, no. C-3006[14]
   24. Potassium chloride (KCL), no. 90330087, UCB[1]
   25. Potassium dihydrogen orthophosphate (KH_2PO_4), no. 10601627[1]
   26. Uranyl acetate $(CH3COO)_2UO_2$.2 aq., no. 2216688[16]
   27. Ethyl alcohol absolute, PA, no. 92287 184[1]
   28. Nitrocellulose (Kollodium) 10%, no. BU 8010 080 09, Balzers[19]
   29. Amyl acetate, no. B 8010 060 15[19]

D. Miscellaneous
   1. Teflon trough (8 cm in diameter)
   2. Ice
   3. Talcum powder
   4. Filter paper
   5. 5% CO_2:95% air mixture, Air Liquide[20]

III. PROCEDURE

Articular cartilage was obtained from the femoral condyles of five human donors (aged between 30 and 64 yr) within 24 h postmortem. Only visually intact cartilage was used. The donors had not received cytostatic drugs or corticosteroids.

A. Culture media
   1. Prepare stock DMEM by adding 5 ml glutamine solution, (5% L-glutamine in distilled water) and 0.5 ml antibiotic/antimycotic solution to 500 ml of DMEM (= DMEM without FBS).
   2. Prepare DMEM by adding 50 ml of FBS to 450 ml of the above-mentioned stock solution (= DMEM with FBS).
   3. Just before culture, add 50 μg of ascorbate/ml of DMEM + FBS and sterilize through Sterivex filter (= culture medium).
   4. Before culture, prepare double-concentrated DMEM:
      —1.338 g DMEM (powdered medium)
      —0.37 g $NaHCO_3$
      —5 mg vitamin C
      in 50 ml distilled water and put through Sterivex filter to sterilize. Add 10 ml of FBS to 40 ml of double-concentrated DMEM.

B. Cartilage digestion
   1. Dice cartilage in DMEM into approximately 1-mm^3 pieces and place them in a sterile spinner flask.
   2. Add 50 ml hyaluronidase (0.2% in DMEM without FBS) per 5-g diced cartilage and keep this mixture for 60 min at 37° C under gentle stirring (approximately 1 rps).
   3. Remove the hyaluronidase solution and replace by 50 ml pronase (0.25% in DMEM without FBS).
   4. Keep the mixture for 90 min at 37° C under constant stirring.
   5. Replace the pronase by 50 ml DMEM with FBS. Store overnight at 37° C.
   6. Replace DMEM by 50 ml collagenase (0.2% in DMEM with FBS).
   7. Keep this mixture for 3 to 6 h at 37° C under constant stirring.
   8. Allow the non-digested fragments to precipitate.
   9. Pour the collagenase solution (which contains the cells) into sterile falcon tubes and centrifuge for 10 min at 100 g.
   10. Remove the supernatant and replace by 1 ml DMEM with FBS.

11. Resuspend the cells and repeat steps 9 and 10 twice (= washing procedure).

C. Setting up the cultures
1. Make a solution of 3% agarose in distilled water and sterilize portions of 5 ml for 15 min in the autoclave. Store at 4° C until use.
2. Melt 3% agarose; coat the bottom of the cryotubes with 100 μl and allow to gel at 4° C.
3. Keep melted 3% agarose at 37° C and mix with an equal volume of double-concentrated DMEM to obtain the desired concentration of agarose (1.5%).
4. Add the chondrocyte suspension to the agarose in the smallest possible volume (approximately 1/10 vol/vol) to a final density of 1.5×10^6 cells per 300 μl.
5. Fill the cryotubes with 300 μl agarose and place them for 30 min at 4° C to allow the agarose to gel.
6. Add 3 ml of culture medium and place the cultures in a moist culture chamber at 37° C with 5% CO_2:95% air.
7. Replace the culture medium twice a week.

D. Solutions for the preparation of aggrecans for electron microscopy (EM)
1. Agarase solution
   a. Dissolve 0.037 g EDTA di sodium salt, 1.31 g aminocaproic acid, 0.072 g benzamidinium chloride and 1.9 g toluene-sulfonyl chloride (= protease inhibitors) in 100 ml PBS.
   b. Add 50 U/ml agarase 3-glycanohydrolase to this solution and store in portions at −20° C.
2. Kollodium solution
   a. Add 3.33 ml of the nitrocellulose solution to 6.66 ml isoamyl acetate
   b. Store in a dark bottle at 4° C.
3. Ammonium acetate solution
   a. Dissolve 4.62 g of ammonium acetate in 10 ml distilled water to obtain a 6 *M* solution.
   b. Dissolve 19 g of ammonium acetate in 1000 ml distilled water to obtain a 0.25 *M* solution.
   c. Store both solutions at 4° C.
4. Tris-EDTA buffer (pH 8.5)
   Dissolve 6.0 g of tris and 1.9 g of EDTA (C.19) in distilled water. Adjust the pH of the solution to 8.5 with HCl. The total volume should be 100 ml. Store at 4° C.
5. Cytochrome C solution
   a. Dissolve 0.19 g EDTA (C.19) in 100 ml distilled water (5 m*M*).
   b. Add 80 ml distilled water to 20 ml of this solution (1 m*M*).
   c. Dissolve 20 mg of cytochrome c in 10 ml of this solution.
   d. Put through Sterivex and store at −20° C in 1 ml portions (Eppendorf tubes).
6. Phosphate buffer to dilute the aggrecans
   a. Dissolve 0.74 g KCl and 0.27 g KH_2PO_4 in 100 ml distilled water (0.1 *M* KCl, 0.02 *M* KH_2PO_4)
   b. Adjust pH to 7.0
7. Staining solution
   a. Prepare a stock solution of uranyl acetate
      —Dilute 5 ml of HCl with 95 ml distilled water to obtain a solution of 5×10^{-2} M HCl
      —Dissolve 0.21 g uranyl acetate in 10 ml of this solution (5×10^{-2} M). Uranyl salts are stabilized in this solution and can be kept at 4° C
   b. Just before staining, pour 10 μl of this stock solution in 10 ml of 90% ethanol.

E. Viscometric studies on agarase-treated hyaluronan samples
1. Dissolve 5 mg of hyaluronan in 10 ml of PBS with the protease inhibitors under C.1.
2. Split the hyaluronan solution in two 5-ml aliquots ("control" and "agarase").
3. Add 0.5 ml of PBS to the control and 0.5 ml of PBS containing 2.5 U of agarase to the agarase.
4. Incubate at 40° C for 24 h.
5. Measure the efflux times of control, agarase, and of a PBS sample with inhibitors through the capillary viscometer.
6. Divide the efflux times of the control and of the agarase by the efflux times of the PBS to obtain the relative viscosity of the control solution and of the agarase-treated hyaluronan solution, respectively.
7. Compare the relative viscosities of both samples.

F. Preparation of aggrecans for EM
1. Digest the agarose gel with the agarase solution at 40° C overnight.
2. Centrifuge the resulting solution at 300 g for 10 min to precipitate the cells. The supernatant contains the liberated aggrecans.
3. Store the supernatant in Eppendorf tubes at −20° C until use.
4. Coat the copper grids as follows:
   —fill a 10-cm petri dish with distilled water
   —apply a drop of the kollodium solution onto the surface
   —remove the kollodium film after 5 min; a very clean water surface is thus obtained
   —again apply a drop of the coating solution onto the surface
   —wait 5 min and apply copper grids onto the formed film; apply a filter paper over the grids and remove it so that the coated grids remain on the paper
   —allow to dry under a lamp.
5. Mix 10 μl of the aggrecan-containing supernatant with 10 μl tris-EDTA buffer, 10 μl 6 *M* NH_4-acetate, 10 μl cytochrome C (2 mg/ml) and 20 μl distilled water in an Eppendorf tube (modification of the method of Kleinschmidt and Zahn, 1959).
6. Place this solution (= hyperphase) on ice.
7. Fill the Teflon holder completely with 0.25 *M* NH_4-acetate (= hypophase).
8. Clean the surface of the hypophase with a glass or Teflon bar.
9. Partially immerse a glass slide (cleaned with alcohol) in the hypophase. The slide is supported by the side of the Teflon holder so that it forms an angle of 30° with the surface of the hypophase.

10. Dust some talcum powder onto the surface at the insertion area of the slide.
11. Slowly release 30 μl of the hyperphase onto the slide, using a micropipette. The hyperphase, containing the aggrecans and cytochrome C, will spread over the hypophase surface and form a monomolecular layer. The boundaries of this layer are indicated by the talcum powder.
12. Touch the surface with a coated grid and immediately immerse in the staining solution (for 60 s).
13. Allow the grids to dry on a filter paper.
14. Select under the EM grids that are not contaminated with dust.
15. Shadow these grids with platinum-palladium (80:20) at an angle of 7° and a distance of 10 cm with a vacuum evaporator.

The grids are ready for electron microscopic investigations.

G. Electron microscopy was performed with a Jeol 1200 EX II, which allows direct measurements on the fluorescent screen, using a special measurement device. Measurements were done at a magnification of 50 000. The following parameters of the aggrecan-aggregates were measured: the number of attached aggrecans per aggregate, the length of the hyaluronan chain, and the length of the attached aggrecans.

IV. RESULTS AND DISCUSSION

The structure of reconstituted aggrecan-aggregates has been extensively studied (Buckwalter and Rosenberg, 1982; Buckwalter et al., 1984, 1985; Front et al., 1989; Kimura et al., 1978; Rosenberg et al., 1970; Rosenberg et al., 1975; Thyberg et al., 1975). Similar studies have not been conducted for native aggrecan-aggregates of cartilage because aggrecan-aggregates in tissues resist extraction with associative solvents. Using these associative solvents, only some 20% of predominantly monomeric aggrecan are extracted from mature hyaline cartilage (Brandt and Muir, 1969). Consequently, tissue extracts with non-dissociative solvents do not allow the study of the entire aggrecan population.

TABLE 1

ELECTRON MICROSCOPIC MEASUREMENTS OF AGGRECAN AGGREGATES SECRETED BY HUMAN ARTICULAR CHONDROCYTES CULTURED IN AGAROSE

		Hyaluronan Length, nm		Number of Attached Aggrecans		Distance Between Attachment Points, nm
Sample	n	x	Range	x	Range	
1_A	25	403	170–1200	15	6–56	27
1_B	25	417	130–1370	16	6–47	26

The agarose suspension culture system enabled us to study native aggrecans synthesized by mature chondrocytes. Chondrocytes cultured in agarose are known to keep their cartilage phenotype, and to synthesize type II collagen (Benya and Shaffer, 1982) and tissue-specific aggrecan (Delbruck et al., 1985). Moreover, in 1.5% of agarose, more than 90% of the aggrecan-aggregates are immobilized in the gel (Verbruggen et al., 1990). In contrast with the associative extraction of aggrecans from tissue samples, the agarase digestion of the artificial agarose matrix makes it possible to obtain almost the whole (85%) aggrecan population (Verbruggen et al., 1990). As opposed to aggrecans extracted from tissues, only de novo and recently synthesized molecules are analyzed in this suspension culture system.

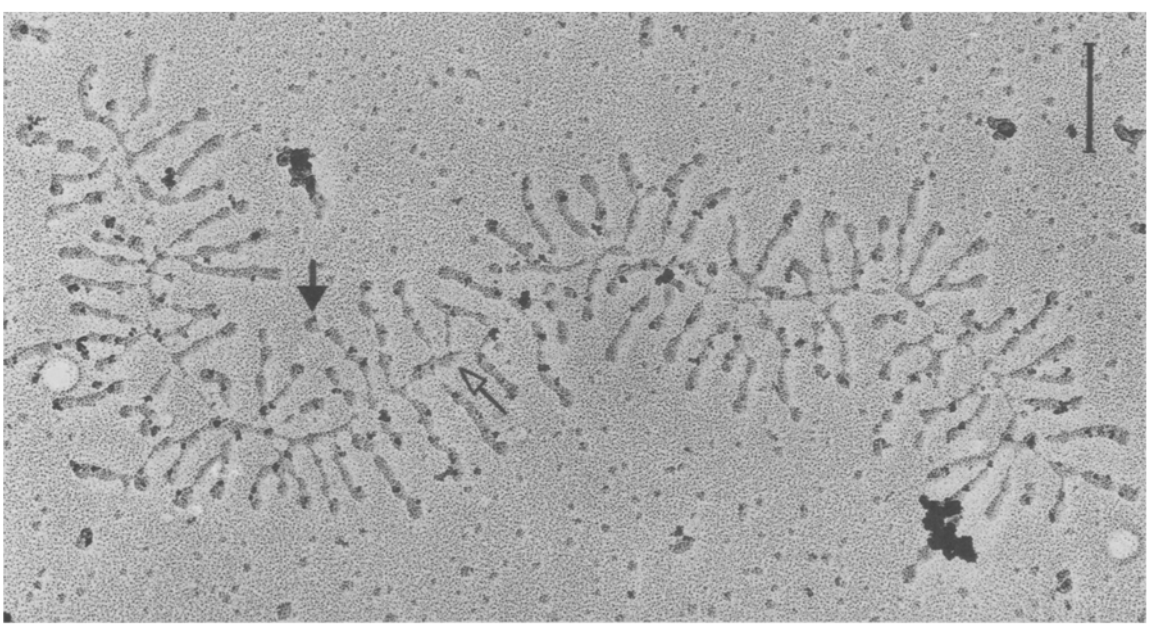

FIG. 1. Electron micrograph of a native, de novo synthesized aggrecan aggregate by human chondrocytes in agarose (*bar* = 200 nm). *Solid arrow* = aggrecan; *open arrow* = hyaluronan chain.

TABLE 2

AVERAGE NUMBER OF ATTACHED AGGRECANS PER AGGREGATE FOR FIVE DIFFERENT SAMPLES[a]

Sample	Donor	Number of Aggrecans/ Aggregate	
		x	range
1	30 M	13	3–105
2	50 M	13	3–103
3	64 M	11	3–109
4	35 M	10	3–95
5	62 F	11	3–73

[a] Age and sex of the donors, average number of aggrecan/aggregate (x), and maximum and minimum number of aggrecans per aggregate (range) are given.

The in vitro-formed native aggrecan-aggregates had the same basic structure as the molecules obtained by both dissociative and associative extraction. They consisted of a central unbranched hyaluronan chain to which several aggrecans were attached. The average length of the attached aggrecans was about 188 nm, which is consistent with the length mentioned by Buckwalter and Rosenberg (1982) for mature bovine nasal cartilage (210 nm) and by Rosenberg et al. (1975) for bovine articular cartilage aggregates (226 nm). For two samples of 25 aggregates from the first donor (age 30 yr), some additional measurements were done. The number of attached aggrecans and the lengths of the hyaluronan chains were measured. Results, together with the calculated distance between the attachment points, are given in Table 1. The distance between the attachment points was found to be 26 to 27 nm and was within the same range as described elsewhere (Buckwalter and Rosenberg, 1982, 1984; Faltz et al., 1972). The electron microscopic appearance of an aggregate is depicted in Fig. 1.

In five samples obtained from five different donors, the number of aggrecans of at least 300 aggrecan aggregates was counted. The average number and the maximum and minimum number of aggrecans per aggregate are given in Table 2. In each sample, the synthesized aggrecan-aggregates were predominantly smaller molecules. Average numbers of aggrecans per aggregate ranged between 10 and 13. About 90% of the aggregates consisted of less than 25 aggrecans. All five samples showed small amounts of aggregates with 30 to more than 100 aggrecans. The size distributions of the aggregates in the molecular populations studied are given in Fig. 2.

Large numbers of non-attached free aggrecans were present in each sample. On the other hand, chains showing free binding sites were not observed. Consequently, hyaluronan chains of de novo synthesized aggrecan-aggregates are maximally loaded with aggrecans and a direct

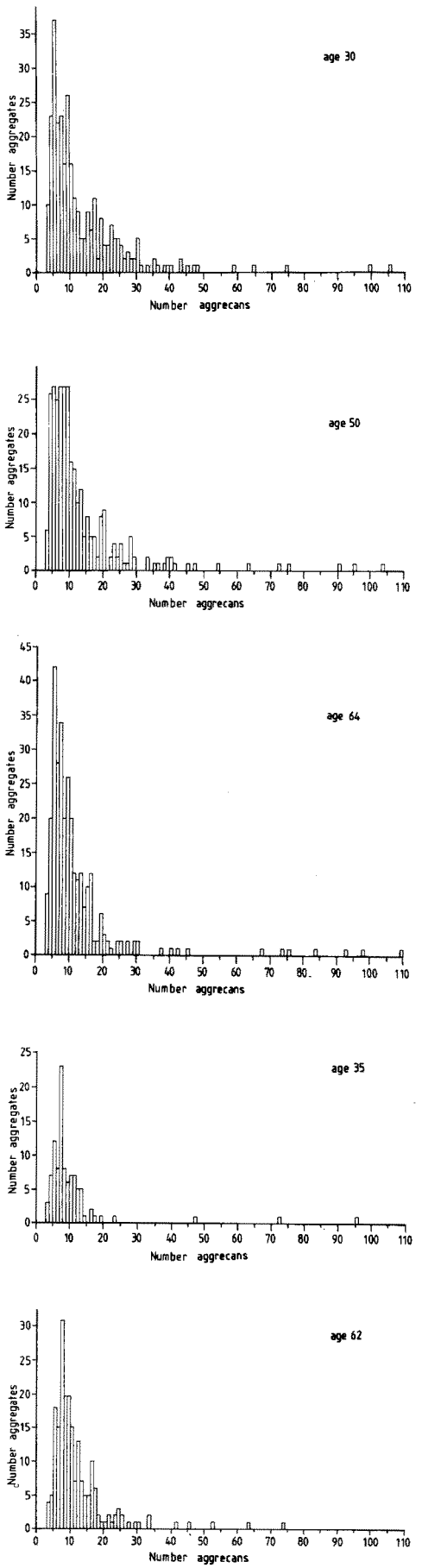

FIG. 2. Histograms showing the distribution of the number of aggregates and the number of aggrecans per aggregate in five samples from five different donors. *Abscissa:* number of aggrecans; *ordinate:* number of aggregates.

relation was found between the number of attached aggrecans and the length of the hyaluronan chain (Fig. 3). Inasmuch as the number of aggrecans largely exceeds the binding sites available on the hyaluronan backbone, it may be concluded that the size of the aggregate depends on the length of the hyaluronan chain.

Viscosity measurements showed that agarase-treated hyaluronan samples had exactly the same relative viscosity as untreated hyaluronan samples. The relative viscosity is proportional to the product of the hyaluronan molecular weight and the concentration in the sample. Hyaluronan concentrations in both non-treated and treated samples were identical. Consequently, no changes in hyaluronan molecular weight were recorded, and we can conclude that agarase, used to solubilize the artificial surrounding matrix, did not affect hyaluronan. Hence, the finding of predominantly smaller aggregates being synthesized by the cartilage cells in this culture system is not an artifact.

The relatively small proportion of very large aggrecan aggregates with up to 100 aggrecans attached to a single hyaluronan chain remains an interesting observation.

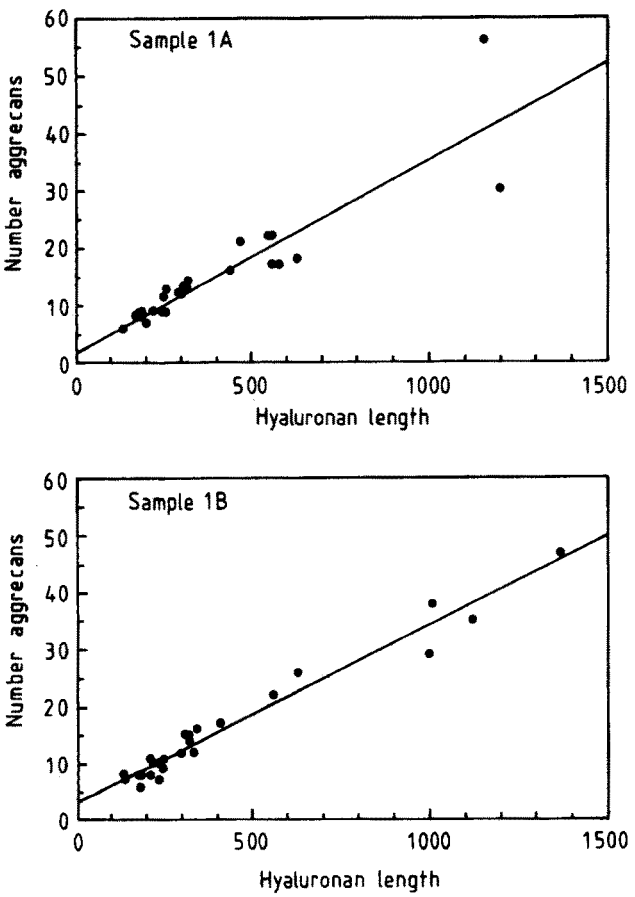

FIG. 3. Correlation between hyaluronan length and number of attached aggrecans. *Abscissa:* hyaluronan length (nm); *ordinate:* number of attached aggrecans. $n = 25$. Linear regression analysis shows a strong correlation between hyaluronan length and number of attached aggrecans for: *A*, $y = 1.77 + 0.0336x$; $r^2 = 0.808$; *B*, $y = 2.99 + 0.0312x$; $r^2 = 0.962$.

Small amounts of such "super" aggregates were also described after centrifugation of aggrecan populations liberated from mature articular cartilage by collagenase digestion (Manicourt et al., 1986). These large aggregates were chiefly observed in immature cartilage tissue (Buckwalter et al., 1985; Buckwalter and Roughley, 1987). The affinity of aggrecans for hyaluronan was found to be increased in osteoarthrotic cartilage (Bayliss, 1986). Therefore, it may teleologically be assumed that these large molecular complexes are involved in the growth and repair of tissues.

We conclude that this culture system offers the possibility to study representative populations of de novo synthesized and native aggrecan-aggregates from mature and phenotypically stable human chondrocytes in vitro.

V. REFERENCES

Bayliss, M. T. Proteoglycan structure in normal and osteoarthritic human cartilage. In: Kuettner K. E.; Schleyerbach, R.; Hascall, V. C., eds. Articular cartilage biochemistry. New York: Raven Press; 1986:295–308.

Benya, P. D.; Shaffer, J. D. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 30:215–224; 1982.

Brandt, K. D.; Muir, H. Characterisation of protein-polysaccharides of articular cartilage from mature and immature pigs. Biochem. J. 114:871–876; 1969.

Buckwalter, J. A.; Rosenberg, L. C. Electron microscopic studies of cartilage proteoglycans. J. Biol. Chem. 257:9830–9839; 1982.

Buckwalter, J. A.; Rosenberg, L. C.; Tang, L. H. The effect of link protein on proteoglycan aggregate structure. J. Biol. Chem. 259:5361–5363; 1984.

Buckwalter, J. A.; Kuettner, K. E.; Thonar, E. J-M. Age-related changes in articular cartilage proteoglycans: electron microscopic studies. J. Orthop. Res. 3:251–257; 1985.

Buckwalter, J. A.; Roughley, P. J. Age related changes in human articular cartilage proteoglycans. In: Transactions 33th Annual Meeting of the Orthopedic Research Society. 12:125; 1987.

Delbruck, A.; Dressow, B.; Gurr, E., et al. In vitro culture of human chondrocytes from adult subjects. Connect. Tiss. Res. 15:155–172; 1985.

Faltz, L. L.; Reddi, A. H.; Hascall, G. K., et al. Characteristics of proteoglycans extracted from the swarm rat chondrosarcoma with associative solvents. J. Biol. Chem. 250:1375–1380; 1972.

Front, P.; Dauguet, C.; Mitrovic, D. R. Effect of cytochrome C concentration on the ultrastructural appearance of bovine nasal cartilage proteoglycans. Stain Technol. 64:113–119; 1989.

Kimura, J. H.; Osdoby, P.; Caplan, A. I., et al. Electron microscopic and biochemical studies of proteoglycan polydispersity in chick limb bud chondrocyte cultures. J. Biol. Chem. 253:4721–4729; 1978.

Kleinschmidt, A. K.; Zahn, R. K. Uber desoxyribonucleinsaure-molekulen in protein-mischfilmen. Z. Naturforsch. 146:770–779; 1959.

Manicourt, D. H.; Pita, J. C.; Pezon, C. F., et al. Characterization of the proteoglycans recovered under nondissociative conditions from normal articular cartilage of rabbits and dogs. J. Biol. Chem. 261:5426–5439; 1986.

Rosenberg, L.; Hellman, W.; Kleinschmidt, A. K. Macromolecular models of proteinpolysaccharides from bovine nasal cartilage based on electron microscopic studies. J. Biol. Chem. 245:4123–4130; 1970.

Rosenberg, L.; Hellmann, W.; Kleinschmidt, A. K. Electron microscopic studies of proteoglycan aggregates from bovine articular cartilage. J. Biol. Chem. 250:1877–1883; 1975.

Thyberg, J.; Lohmander, S.; Heinegard, D. Electron-microscopic studies on isolated molecules. Biochem. J. 151:157–166; 1975.

Verbruggen, G.; Veys, E. M.; Wieme, N., et al. The synthesis and immobilisation of cartilage-specific proteoglycan by human chondrocytes in different concentrations of agarose. Clin. Exp. Rheumatol. 8:371–378; 1990.

The technical assistance of R. Maenhout is gratefully acknowledged. A. M. Malfait is an Nationaal Fonds Wetenschappelyk Onolerzoek (Belgium) research assistant. This work was supported by European Economic Community-BRIDGE grant BIOT-0196 and by NFWO grants 3.0077.99 and 9.850588.

[1] Vel, Leuven, Belgium
[2] Dupont, Wilmington, DE
[3] Billups-Rotenberg, Del Mar, CA
[4] Bellco Glass Inc., Vineland, NJ
[5] Mettler Instrumente AG, Greifensee, Switzerland
[6] Jeol Europe, Brussels, Belgium
[7] Laborimpex, Brussels, Belgium
[8] Schott, Mainz, Germany
[9] Becton-Dickinson Laboratory Systems, Dublin, Ireland
[10] A/S Nunc, Roskilde, Denmark
[11] Millipore Products Division, Bedford, MA
[12] Analis, Namur, Belgium
[13] GIBCO Laboratories, Gent, Belgium
[14] Sigma Chemical Co., St. Louis, MO
[15] Fluka, Buchs, Switzerland
[16] E. Merck, Darmstadt, Germany
[17] Janssen Chimica, Geel, Belgium
[18] Fidia, Abano Terme, Italy
[19] Balzers Union Aktiengesellschaft, Liechtenstein
[20] Air Liquide, Liege, Belgium