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Chondrocyte behavior in fibrin glue in vitro

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To test fibrin glue as a vehicle in chondrocyte transplantation, chondrocytes, isolated from articular cartilage of young rabbits, were mixed with Tissucol®, a highly concentrated fibrin glue, and cultured for 7 days. Histology, autoradiography (35-S) and electron microscopy were used to study chondrocyte behavior and phenotypic expression. Chondrocytes multiplied, retained their morphology, and produced matrix in fibrin glue as long as the cells were surrounded by the glue. Glue disintegration started after 3 days and was accelerated by higher cell concentrations.

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Full thickness articular cartilage defects heal spontaneously by the formation of fibrous or fibro-cartilaginous tissue, of relatively poor biomechanical quality (Mitchell and Shepard 1976, Furukuwa et al. 1980, Nelson et al. 1988, Homminga et al. 1989). Repair of lesions with isolated chondrocytes entails problems with graft fixation (Bentley et al. 1978) or rejection in case of allografting (Elves 1974). Biological substances, like collagen gel (Kimura et al. 1983, Wakitani et al. 1989), hyaluronic acid (Robinson et al. 1990) and fibrin glue (Itay et al. 1987) have been used in chondrocyte transplantation for the repair of small chondral defects in animal experiments. The efficacy of these delivery agents depends on biocompatibility, cytotoxicity, biodegradability and the ability to offer a good fixation to subchondral bone and host cartilage. Optimal delivery substances combine an appropriate rate of biodegradability with the capacity of the cells to multiply and produce matrix components. Particularly the latter capacity is important in allografting to avoid immunological responses and subsequent rejection (Heyner 1969, Elves 1974, Malseed and Heyner 1976).

To create a cartilaginous tissue in vitro for repair of chondral defects, knowledge has to be gained about the behavior of chondrocytes in delivery substances during culture experiments. It is well known that chondrocytes behave differently depending on the culture conditions. The cells dedifferentiate in monolayer but retain their phenotype in high density cultures (Coon 1966), collagen gel (Kimura et al. 1983) and agarose (Buschmann et al. 1991). Particularly the rate of cell division, matrix production and degradation of the vehicle are important variables.

We tested and optimized the use of Tissucol®, a highly concentrated human fibrin glue, for in vitro growth of chondrocytes.

Material and methods

1 rabbit was used for each cell culture experiment. Cartilage was harvested in small slices with a sharp knife under sterile conditions from the knee joints of New Zealand rabbits, age 6 weeks. The slices were cut into small pieces and incubated in RPMI culture medium (1640, Flow Labs; supplemented with 2 mM L-glutamine, 1 mM pyruvate, and 1.2% gentamicin) with 100 I.U./mL collagenase (Worthington Biochemical Corporation) for 16 h, at 37 °C, with 95% air and 5% CO_2. After 16 h in collagenase, the pieces of cartilage were almost completely digested. The supernatant was collected and the cells were washed 3 times by centrifugation and resuspension in 20 mL RPMI. Total cell counts and viability were assessed in a hemocytometer after staining with trypan blue. The cells were centrifuged and resuspended to obtain the appropriate cell concentrations.

The fibrinogen component was mixed with 3000 KIE/mL aprotinin and thrombin 4 I.U. in a 40 mmol/mL solution of calcium chloride. Both solutions were heated to 37 °C while the fibrinogen vial was placed in a stirring device until it was dissolved. Of the chondrocyte suspension 100 µL was homogeneously mixed with the thrombin/$CaCl_2$ solution. In a special device the fibrinogen solution was mixed with the thrombin/$CaCl_2$/cell suspension. Samples of 0.1

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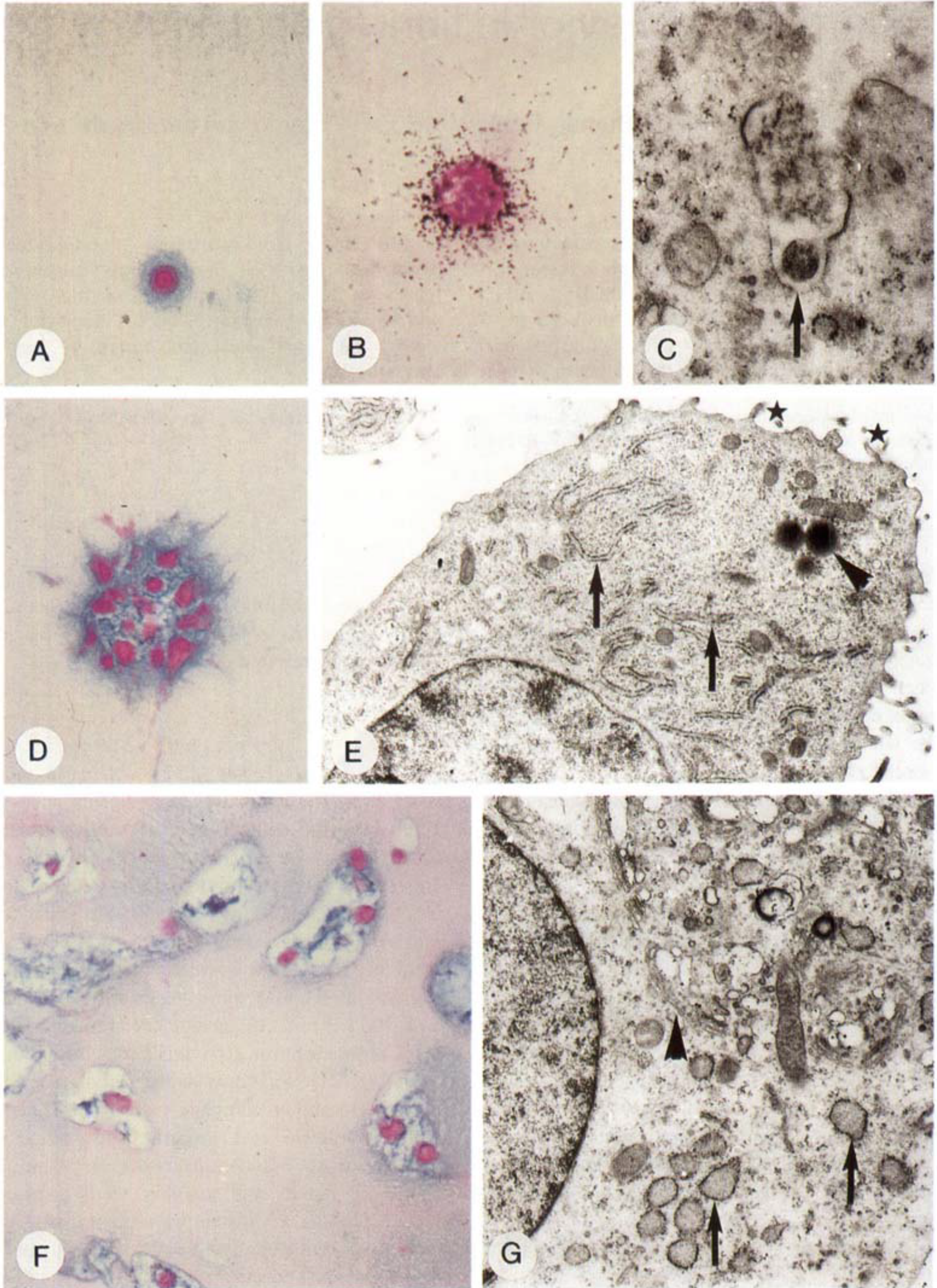

Figure 1.

A Chondrocyte in the glue, Day 2, ×350.
B Autoradiograph after labeling with 35S-sulfate. Note many silver grains around the chondrocyte, ×1200.
C Exocytosis (arrow) of secretory material, 2 days after the start of the culture, ×25 000.
D Cluster of chondrocytes surrounded by alcian blue positive matrix, cultured for 3 days, ×350.
E Ultrastructure of chondrocyte after 2 days in the glue. Note rough endoplasmic reticulum, (arrows), small protrusions of the plasmalemma (asterisks), and lipid droplets (arrow head), ×8000.
F Clusters of cells, located in the glue that is partly dissolved, Day 5, ×350.
G Ultrastructure of chondrocyte after 5-day culture. Note Golgi zone (arrowhead), and dilated cisternae of rough endoplasmic reticulum (arrow), ×12500.

Table 1. Number of cells, surface area (percentages) of matrix and empty space after culturing 10^6 cells per mL. Mean *SD*

	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
Cells	47	*14*	41	*7.2*	36	*4.5*	67	*10*	101	*22*	154	*20*	215	*30*
Percent cells in clusters	0		0		12	*4*	47	*12*	76	*23*	85	*13*	92	*34*
Number of cells in clusters					2.3	*1.2*	3.5	*1.7*	4.5	*2.1*	6.0	*3.5*		
Surface area of matrix	0		1		1		2		3		7		14	
Surface area of dissolved glue	0		0		0		2		5		18		40	

mL containing 10^4, 5×10^4, 10^5, 5×10^5, 10^6 and 2×10^6 cells per mL were sampled to the culture discs, and 10 min later, after the glue had coagulated, supplemented with culture medium to which 10% fetal calf serum was added. Every other day the medium was changed. For autoradiography, the cultures were incubated for 24 hours in medium containing 20 mCi/mL 35S-sulphate. Subsequently, the cultures were rinsed to remove free sulfate.

From Days 1–7, samples were harvested daily and fixed in 0.1 M phosphate buffered (pH 7.4) solution of 1% paraformaldehyde and 1.25% glutaraldehyde for 48 hours and embedded in polymethylmethacrylate. For routine histology, histomorphometrical studies, and for the assessment of matrix production, 7-μm thick sections were stained with hematoxylin and eosin (HE) or with alcian blue. For autoradiography, sections were dipped in Ilford L4 photographic emulsion, dried, stored in the dark at 4 °C and developed after 2 weeks with amidol and counterstained with HE.

For electron microscopy, pellets were postfixed in 1% OsO_4 in the buffer and embedded in Epon 812. Semi-thin sections (2 μm) were counterstained with toluidine blue. Adjacent ultra-thin sections were mounted on oval 2-mm 1-hole grids, stained with uranyl acetate and lead citrate, and examined in a Philips 301 electron microscope.

Histomorphometric analysis of the specimens included cell-counting and assessment of glue dissolution in the light microscopic sections with an eyepiece grid placed in the ocular, in a surface area of 0.90 mm, according to the procedure described by Gundersen (1977).

Results

After 16 hours in collagenase, the cartilage was completely digested; the number of viable cells, as assessed after trypan blue staining, was 99%. The cells were homogeneously dispersed throughout the glue. Only occasional clusters of cells were present, probably because of incomplete resuspension of the cell pellets. No matrix was present around the cells.

After 1 day the gross appearance of the chondrocyte-fibrin glue suspension was unchanged, i.e., a white semi-solid mass. Light microscopically, all cells had retained their round shape, showing no sign of cell division or matrix production. Ultrastructurally, the morphology of the cells had changed slightly. Fibrin fibers were observed in indentations of the cell's membrane, suggesting a close contact of the glue and the cell. Some of the cells contained large homogeneous lipid aggregates (Figure 1E).

After 2 days the first light microscopic changes were noted. A majority of the cells were surrounded by an intense alcian blue positive area (Figure 1A). Autoradiography showed silver labeling at the same location indicating that sulfated substances were secreted by the cells (Figure 1B). Ultrastructurally, most cells appeared to contain several Golgi zones and large areas of rough endoplasmic reticulum, partly filled with dense secretory material (Figure 1E, G). Omega-shaped membrane indentations of the plasma membrane containing electron-dense material, indicated active secretion of the contents of secretory products to the extracellular space (Figure 1C). Occasionally cells were present that had cytoplasmic protrusions. Light microscopic cell-clustering appeared to be rare; the majority of the cells were lying alone. Some of the cells were surrounded by a white halo in which no matrix staining appeared, indicating pericellular glue dissolution.

After 3 days cell clusters were present (Figure 1D). Occasionally metaphase figures were found as a sign of cell division. Particularly around the cell clusters the fibrin glue was dissolved, but the same phenomenon could also be observed around individual cells. Cells located at the surface of the fibrin clot had an elongated appearance. Overall, alcian blue staining had become more intense in the vicinity of the cells.

At Day 4 the overall impression was comparable to Day 3 but more pronounced. The cell clusters were larger, the dissolution of the glue had progressed (Table 1). Ultrastructurally, the cells contained large

areas of rough endoplasmic reticulum, filled with secretory material (Figure 1G). Within some of the clusters, the cell shape had changed from oval to spindle-like and matrix production had decreased. Especially around the dedifferentiating cells there were lucent zones. The ultrastructural appearance of these dedifferentiating cells was the same but showed little signs of active secretion.

At Day 5–7 the chondrocyte cluster became larger, and glue dissolution and cell dedifferentiation progressed (Figure 1F). Particularly the fibroblastic cells showed many metaphase figures. From Day 4–Day 7 specimens with various cell concentrations, ranging from 10^4 to 2×10^6 cells/mL, showed increasing differences: at Day 7, cell number ranged from a mean of 40 in the lower concentrations to a mean of 215 in the highest (Table 1). After 7 days the average area of glue dissolution was 8% in the 10^4 cell/mL specimens and 40% in the 2×10^6 cell/mL specimens (see Table 1 for histomorphometric data for the 10^6 cell/mL concentration). From Day 7 the fibrin clot started to fall apart and finally disintegrated completely.

Discussion

Tissucol®, a highly concentrated human fibrin glue, is a potentially suitable biological vehicle for chondrocyte transplantation by its proven biocompatability (Scheele and Pesch 1982), biodegradability (Staindl and Galvan 1982) and binding capacity to subchondral bone (Keller et al. 1985). However, the in vivo study of chondrocyte transplantation in fibrin glue by Itay et al. (1987) showed only limited ability to repair cartilage lesions. It was suggested that fibrin glue may be cytotoxic. In this culture study no adverse effects of fibrin glue on chondrocytes were observed: they retained their phenotypic expression as long as they were surrounded by the glue. Moreover, the cell number increased during culture, resulting in the formation of large chondrocyte clusters.

From the results of this study it may be concluded that chondrocytes are active in terms of cell division and matrix production when cultured in fibrin glue. A longer time in culture makes the chondrocyte/fibrin glue mixture unsuitable for transplantation, since cells start to dedifferentiate and the homogeneity of the substance is lost due to disintegration of the glue. The suitable period of time seems to depend on initial cell concentration. Biodegradability of the vehicle is a must in chondrocyte transplantation, but too rapid degradation may influence cell adherence and integration in a negative way. Degradation of the substance must keep pace with cell division and matrix production, giving temporary support to the chondrocytes for its organization and maturation into hyaline cartilage. Fibrin glue may be suitable for chondrocyte transplantation, but definite conclusions can only be drawn after in vivo studies.

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