Perichondrium-Wrapped Collagenous Matrices to Induce Chondroneogenesis

An In Vitro Study

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Objective: To develop a model for cartilage regeneration in vitro, to be used for cartilage reconstruction in vivo.

Methods: Collagenous matrices were wrapped in a perichondrium layer. The matrices served as carriers to allow migration of cells from the perichondrium into the matrix. Culture conditions stimulated cell growth and proliferation.

Results: After 4 weeks of culturing, microscopic examination showed an increase of cell layers around the matrices but also of cells migrated into porous matrices. Immunohistochemical staining of these cultured cells expressed type II collagen intracellularly.

Conclusions: This model seems appropriate to culture mucoperichondrial explants in combination with collagenous matrices. Cells migrate into the pores of the matrix, survive, and synthesize matrix components. Actual formation of cartilage has not been shown to occur. Adding growth factors to this model may influence induction of this activity.

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In functional-aesthetic surgery of the nose, a variety of different implant materials are in use. These implants are essential in closing cartilaginous defects (eg, septum perforation), augmentation of the nose (eg, onlay graft of the nose dorsum), and reconstruction of the contour of the nose (eg, cleft palate syndrome). Alloplastic materials have been developed, such as silicone, porous polyethylene, porous polytetrafluoroethylene-carbon, and porous polytetrafluoroethylene, of which the last one is most frequently used currently. These materials are biocompatible but not completely without reaction of the host.1,3

Allogeneic or xenogeneic implants, such as irradiated bone or cartilage, are readily accepted by the host and show minimal tissue reaction. On the other hand, long-term follow-up shows a tendency for their gradual resorption.4,5 Also frequently used is autogenous cartilage. It can be obtained from the nose (nasal septum), and in elaborate reconstruction it can be harvested from the ear (conchae cartilage) or rib (costal cartilage). It is the implant material of first choice, despite the morbidity of the donor site.3 This morbidity is the reason that numerous research groups are searching for alternative implant materials. Bean and coworkers6,7 have shown that demineralized bovine bone matrix can be transformed into a suitable cartilage substitute by exposing this material to a vascularized perichondrial flap.6,7 In vitro studies by Bujia et al8-9 showed that it is possible to obtain a 3-dimensional cartilaginous structure from polylactic acid fleeces and human chondrocytes in agarose gel. Kim et al10 engineered nasoseptal cartilage replacements through a process of neomorphogenesis of cartilage by culturing bovine chondrocytes in close relation to biodegradable polymers composed of polyglycolic acid and poly-L-lactic acid. However, an implant that is easily obtained with all the benefits of autogenous cartilage has yet to be found.

The goal of the present study was to find a model for cartilage regeneration in vitro.8-9,11 For this purpose, different collagenous matrices were wrapped in a perichondrium layer.7,12,13 The matrices serve as a carrier for migration of cells from the perichondrium into the matrix. Optimal culture conditions should stimulate cell growth and proliferation. After some time, the obtained matrix in vitro will be used for reconstruction in vivo. The implanted carrier will resorb gradually5,14,15 and subsequently be replaced by the newly formed matrix.
MATERIALS AND METHODS

MATERIALS

The following materials were purchased for this study: Dulbecco modified Eagle medium, fetal calf serum, glutamine, and phosphate-buffered saline (Gibco, Merelbeke, Belgium); gentamicin sulfate (Centrafarm, Etten-Leur, the Netherlands); penicillin G sodium (Yamamoto, Leiderdorp, the Netherlands); ascorbic acid and 1% bovine serum albumin (BSA) (Sigma-Aldrich Corp, St Louis, Mo); petri dishes (Greiner, Frickenhausen, Germany); 12- and 24-well plates and low-affinity well plates (Costar, Cambridge, Mass); goat anti–bovine type II collagen and fluorescent isothiocyanate–conjugated rabbit antigoat antibody (Southern Biotechnology Associates Inc, Birmingham, Ala); mounting medium (Vectashield; Vector Laboratories Inc, Burlingame, Calif); and tritiated proline (937 mBq/mL) (New England Nuclear, Boston, Mass).

TISSUE CULTURE

Mucoperichondrium was obtained from the nasal septum of adult rabbits (female New Zealand white, 24 weeks; Charles River, Someren, the Netherlands)17,18 Fentanyl citrate, 0.315 mg/mL, with flunisone, 10 mg/mL (Hynnorm; Janssen Pharmaceutica, Beerse, Belgium), was used to anesthetize the rabbit before killing; the nasal septum was excised in toto and divided into 3 equal parts (7 × 10 mm). From each part, the mucoperichondrial layer was removed from both sides with a perichondrium elevator. The mucoperichondrium was rinsed 4 times with phosphate-buffered saline (PBS). In a petri dish, a matrix was presoaked with medium and the mucoperichondrial layer was wrapped around this matrix with the perichondrial part of the tissue facing the matrix. The mucoperichondrium was attached to the selected matrix with a small hemoclip (Ligaclip; Johnson & Johnson, Amersfoort, the Netherlands) and transferred to culture plates. For each experiment the number in 1 group was 4.

The medium consisted of Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, 1% glutamine, gentamicin sulfate (50 µg/mL), penicillin G sodium (50 µg/mL), and ascorbic acid (50 µg/mL). After fixation of the perichondrium to the matrix, the medium was refreshed every half hour for 2 hours. After this, the medium was refreshed 3 times a week. The well plate was stored in a humidified incubator at 37°C in an atmosphere of 5% carbon dioxide and 95% air-dried and frozen at −20°C.

After preparation of the mucoperichondrium, the perichondrial layer was very dense and 5 to 6 layers thick. After finishing the culture (as described by Everts and Beertsen20), the perichondrial part of the tissue facing the matrix, the medium was refreshed every half hour for 2 hours. After this, the medium was refreshed 3 times a week. The well plate was stored in a humidified incubator at 37°C in an atmosphere of 5% carbon dioxide and 95% air (Stericult 200; Brouwer Scientific Inc, De Meern, the Netherlands). Tissue was fixed in a solution of 4% paraformaldehyde in 0.1-mol/L PBS (pH 7.4), dehydrated, and embedded in glycol methacrylate. Sections were made of the mucoperichondrium-wrapped matrices and stained with hematoxylin-eosin. The cells on the bottom of the well plate were fixed as indicated above and stained directly in the wells.

 IMMUNOLOCALIZATION

After the culture period, the tissue was rinsed 4 times with PBS and fixed for 5 minutes in 75% ethanol. The tissue was air-dried and frozen at −20°C.

Cryosections were made for immunohistochemical staining with an antibody against type II collagen. For this purpose, sections were incubated first with 1% BSA in PBS (BSA/PBS) to block nonspecific binding (room temperature, 30 minutes). The sections were washed with 0.05% PBS-Tween (3 times, 15 minutes each time). The antitype II collagen was diluted 1:250 in BSA/PBS, added to the cryosections, and incubated for 90 minutes at room temperature. The sections were rinsed with PBS-Tween (3 times, 15 minutes each time). Fluorescein isothiocyanate–conjugated rabbit antigoat antibody was diluted 1:500 in BSA/PBS and the sections were incubated for 60 minutes (room temperature). The nuclei were stained with propidium iodide diluted in 1:100 PBS for 10 minutes. Finally, the sections were rinsed with PBS-Tween (3 times, 15 minutes each time). The sections were covered with mounting medium (Vectashield).

PROLINE INCORPORATION

Tritiated proline (0.74 MBq per well) was added to explants 24 hours before the tissue was collected. The tissue samples and wells were washed 3 times with PBS, then 300 µL of 1N sodium hydroxide was added and kept for 20 minutes at room temperature. The cells were homogenized and 100 µL was taken out and analyzed in the scintillation counter (Wallac 1450 Microbeta Plus; Wallac, Turku, Finland). Autoradiography of the tissue explants was performed by adding tritiated proline in the last 24 hours before finishing the culture (as described by Everts and Beertsen20).

RESULTS

TISSUE CULTURE

After preparation of the mucoperichondrium, the perichondrial layer was very dense and 5 to 6 layers thick. In culture this layer lost its dense scaffold, and in the first days of culturing a number of cells appeared to die.
This was assumed because of the turbid yellowish appearance of the medium. After the first refreshment of the medium, the turbid yellowish appearance was no longer seen. After the second refreshment of the medium, cells were seen to migrate to the bottom of the well plate. During the next weeks of culturing, the numbers of these cells increased considerably, finally occupying almost the entire bottom of the well. Microscopic examination of the explants showed that, compared with 2 weeks of culturing, at 4 weeks the thickness of the perichondrial layer around the matrix had increased from 6 to 9 cell layers (Figure 1). The space between the matrix and the mucoperichondrium was filled up with cells, which originated from the perichondrium.

**MATRICES**

Cells from the perichondrial layer migrated into some of the matrices. This was only apparent in matrices with pores; in the nonporous dental and cartilaginous matrices, such a migration of cells was absent. This migration was more pronounced after 4 weeks of culturing than that after 2 weeks (Figure 2). The number of cells that had migrated into matrices containing elastin was similar to the number found in cross-linked matrices.

No differences in cellular morphologic characteristics were found between mucoperichondria in contact with type II or type I collagenous matrices. In an attempt to increase migration of cells in collagen type II matrices, holes were burred in these matrices. Despite this treatment, the porosity of the matrices remained very low compared with that of dermal bovine type I collagen matrix, and an increase in migration was not seen. In addition, epiphyseal plate of bovine bone was tested because of the presence of naturally formed canals in this matrix. Since a tight fixation of the mucoperichondrium to the matrix was not possible because of the irregularity of this matrix, migration of cells into this matrix could not be evaluated well. Up to 4 weeks of culturing, in this in vitro model, no resorption or disintegration (except for commercially available demineralized bovine bone matrix [Osteovit]) of all the used matrices could be identified. Taken together, the dermal bovine type I collagen matrix proved to best suit our goals and was selected for use in subsequent experiments.

Since numerous cells appeared to migrate from the mucoperichondrium to the bottom of the wells and not into the matrices, stimulation of migration into the matrix was attempted by (1) placing the explants on a grid or (2) using low-affinity well plates. Under these conditions, however, an increased invasion into the matrix was not found. So far, no statistical analysis has been performed on these attempts.
IMMUNOHISTOCHEMISTRY AND PROLINE INCORPORATION

After culturing for up to 4 weeks, the morphologic structure of the cells attached to the bottom of the wells had changed from an elongated fibroblastlike shape to a more rounded one. Immunohistochemical staining of these cells with anti–type II collagen showed some expression of this protein intracellularly. In the extracellular space, type II collagen could not be detected (Figure 3).

Analysis of the incorporation of tritiated proline showed a time-dependent increase for cells on the bottom of the wells (Figure 4). An increased incorporation was also found in the explants, but this was only apparent at the 1- and 2-week intervals (Figure 5).

Autoradiography of these explants showed that cells present in the mucoperichondrium had incorporated the labeled amino acid. In addition, labeled cells were found in the matrix. Some of the label proved to be incorporated in the extracellular matrix (Figure 3).

COMMENT

The observations presented in this study indicate that mucoperichondrium tissue explants can be used to wrap a collagenous matrix and that the cells of this tissue survive. Our data show that a fraction of the cells migrate into the matrix, whereas other cells migrate out of the tissue onto the bottom of the wells. During the first few days of culturing, some cells die, probably because of manipulation of the tissue. At later intervals, an increased thickness of the perichondrial tissue was observed, indicating growth of the tissue. This finding was in line with the incorporation data, which demonstrated that protein synthesis continued during culturing. In none of the experiments were cytotoxic effects of the matrices observed.

Migration of cells into matrices occurred only in those containing pores. In the nonporous dental and cartilaginous matrices, no ingrowth of cells was found. Our data suggest that a matrix with pores is essential to finally achieve our goal: remodeling of an existing matrix into an implantable mold.

To prevent outgrowth of cells on the bottom of the wells and thereby, perhaps, stimulate these cells to migrate into the pores of the matrix, experiments were performed by culturing the mucoperichondrium-wrapped matrix on a grid or on a low-affinity well plate. Under these conditions, however, an increased migration into the matrix was not found.

The morphologic structure of the cells on the bottom of the wells changed during prolonged culturing from an elongated fibroblastlike shape to a more rounded one. The rounded shape of the cells may suggest that mucoperichondrial cells differentiated into a chondrocyte phenotype. Staining of these cells with an antibody against type II collagen supported this option. Type II collagen could not be visualized in the extracellular space, however. We assume that the collagen was secreted in the medium and not deposited on the bottom of the wells.

In conclusion, this model seems appropriate to culture mucoperichondrial explants in combination with collagenous matrices. Cells migrate into the pores of the matrix, survive, and synthesize matrix components. Actual formation of cartilage has not been shown to occur, but adding growth factors to this model may induce such an activity. With the use of mucoperichondrial cells instead of matured chondrocytes, these cultured cells could probably be more easily induced by growth factors to stimulate growth and differentiation to produce cells closely related to chondrocytes.
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REFERENCES


Clinical Implications

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