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Engineering of Cartilage Tissue Using Bioresorbable Polymer Fleeces and Perfusion Culture

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Replacement of injured or diseased skeletal tissues by either autograft or allograft cartilage has increased steadily during recent decades. The ideal method is to use autologous cartilage; however, this is extremely limited due to the scarcity of donor sites. We present a new approach to the *in vitro* formation of cartilage grafts for autologous grafting in reconstructive surgery. Bioresorbable polymer fleeces of polylactic acid were used as temporary cell carrier matrices to establish three-dimensional cultures of human chondrocytes. The polymer surface was coated with poly-L-lysine before cell integration. These cell-polymer tissue constructs were encapsulated with low melting point agarose and then placed in perfusion culture chambers to provide a constant supply of nutrients into the cultures. The culture medium consisted of Ham's F12 supplemented with 2% fetal calf serum and 50 μg/ml ascorbic acid. The cell-polymer tissues were harvested and frozen for toloudine and alcian blue staining as well as electron microscopic examination after different periods of time in culture. A monoclonal antibody specific for collagen type II was used to characterize the cell phenotype. With this culture procedure chondrocytes maintained a differentiated phenotype with synthesis of collagen and proteoglycan. Collagen fibrils with clear cross-striation were evident in electron microscopic images. The results show that our organotypic cell culture method allows the *in vitro* production of bioartificial cartilage for transplantation. *Key words: cartilage grafting, chondrocytes, tissue engineering.*

INTRODUCTION

The limited supply of fresh autologous cartilage tissue for use in reconstructive surgery necessitates the use of vital banked allografts. These cartilage fragments often degenerate, leading to the formation of fibrous tissue (1, 2).

Cartilage tissue is characterized by chondrocytes and an abundant extracellular matrix containing type II collagen and proteoglycans. When cartilage cells are isolated by enzymatic digestion and cultured as a monolayer, they dedifferentiate by losing their rounded shape and acquire a fibroblast-like appearance. Moreover, their collagen synthesis is switched from type II to type I. However, when chondrocytes are cultured in agarose they show a more stable phenotype, and gel cultures are even able to induce redifferentiation of dedifferentiated chondrocytes. Agarose culture has therefore provided the possibility of promoting the *in vitro* formation of cartilage tissue (3, 4).

The numbers of cartilage cells can be increased by serial subculture as a monolayer, in which they lose their differentiated phenotype. This functional state can be regained by transferring the cells into an appropriate carrier medium such as an agarose gel. The behavior of chondrocytes should make it possible to prepare sufficient amounts of transplantable cells from small autologous biopsies (5).

We have used gel-like substances in attempts to synthesize histologically living cartilage tissue *in vitro* (6). A different approach involves resorbable sutures or non-woven meshes as carriers for chondrocytes *in vivo* (7). With gels like agarose, a sufficiently, homogeneous three-dimensional (3-D) cell suspension can be obtained and cultured; however, these gel cultures lack the mechanical stability and behavior required for transplants in reconstructive surgery. Therefore, two prerequisites are necessary for the engineering of a vital transplant. First, the 3-D cell carrier must fulfil structural requirements, but must also be resorbed gradually after implantation. Second, sufficient preformation and differentiation of a cartilage-like intercellular matrix must be achieved *in vitro* before implantation to avoid the formation of fibrocartilage *in vivo*.

The aim of this work was to study the possible use of a bioresorbable polymer fleece with a large internal surface area as a temporary matrix to establish 3-D cultures of isolated human chondrocytes.

MATERIAL AND METHODS

Isolation of chondrocytes

Normal nasal cartilage tissues were obtained from patients during reconstructive surgery and processed

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as previously described (5). Briefly, the extracellular matrix was digested for 12–18 h at 37°C in the presence of 2 mg/ml type II collagenase (Seromed, Berlin, Germany), 0.1 mg/ml hyaluronidase (Serva, Berlin), and 0.15 mg/ml DNase (Paesel, Frankfurt, Germany) in RPMI 1640 medium (Seromed). The resulting cell suspension was filtered through a nylon sieve with a pore size of 88 μm and washed 3 times in phosphate buffered saline (Seromed).

Three-dimensional culture

Bioresorbable polymer fleeces of poly-L-lactid (Dr. Planck, Institute for Textil Research, Denkendorf, Germany) were used. The polymer surface was coated with poly-L-lysin (Seromed) to support cell attachment. The biomaterials containing the cells were encapsulated with 4% low melting point agarose solution (Sigma, Deisenhofen, Germany). The prechilled agarose was solidified within a few minutes by cooling it in cold water. The resulting cell-polymer tissues were cultured in perfusion culture chambers (Minucells and Minutissue, Bad Abbach, Germany) to provide a constant supply of nutrients by diffusion. In general, the pump cartridges were adjusted to deliver 1 ml/h of Ham's F12 culture medium (Seromed) supplemented with 2% fetal calf serum and ascorbic acid (Fig. 1). The pump was operated with on/off intervals of 30 min throughout the entire culture period.

Samples of the culture were prepared as 4 to 5 μm-thick cryostat sections for the identification of cell phenotype and matrix synthesis with light microscopy. Specimens were also prepared for transmission electron microscopic examination.

Identification of cell phenotype

The differentiation state of the chondrocytes was examined using a specific monoclonal antibody directed against human type II collagen (Chemicon, Tamecula, CA, USA). The monoclonal antibody was used with an alkaline phosphatase/anti-alkaline phosphatase (APAAP) method as previously described (6).

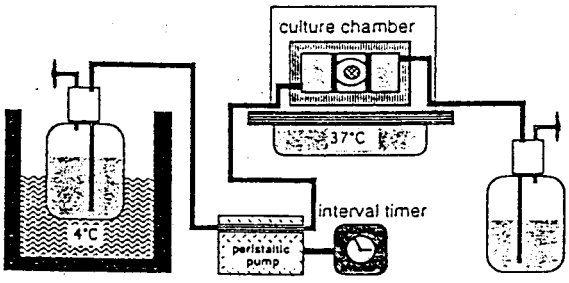

Fig. 1. Perfusion culture system.

Matrix synthesis

Azan staining for proteoglycan synthesis and Alzian-blue staining for collagen synthesis were used on cryostat sections (9).

For electron microscopic examination the specimens were fixed after perfusion culture for 20 min in medium containing 3% glutaraldehyde. After the initial fixation step, the specimens were post-fixed with 1% osmium tetroxide in phosphate buffered saline and dehydrated in a series of alcohols, passed through propylene oxide, and embedded in Epon. Semithin sections were stained with Richardson solution and examined under a light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate.

RESULTS

With the culture procedures described, the chondrocytes rapidly adhered onto the bioresorbable carrier fleece. The chondrocytes in cell-polymer cultures continued to synthesize collagen and proteoglycans, as shown by histochemical staining on cryosections. Positively stained zones were not only limited to the proximity of chondrocytes, but apparently filled spans between more distant cells and fibres. Futhermore, abundant collagen fibrils with typical periodical fibrillar staining were clearly visible near the cells after 1 week in culture (Fig. 2). Immunohistochemi-

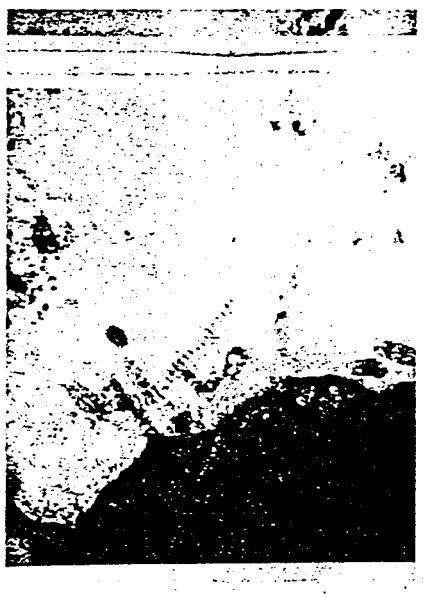

Fig. 2. Transmission electron microscopic image of chondrocytes cultured for 14 days. Near the chondrocytes newly synthesized collagenous networks are found in close contact to the chondrocyte protrusions. Collagen is recognizable by its typical periodic staining.

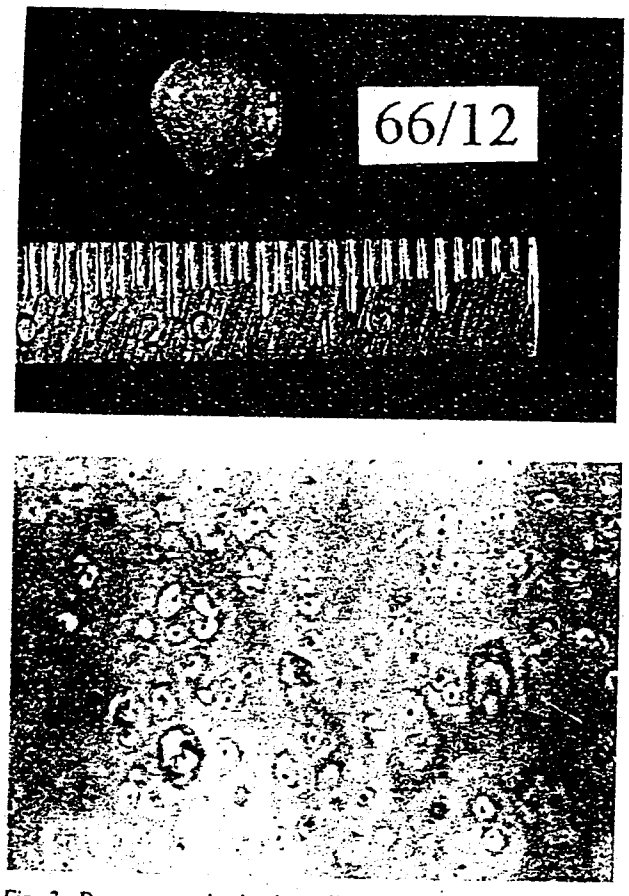

Fig. 3. De novo synthesized cartilage (top) shows clusters of chondrocytes-like cells surrounded by a new matrix (bottom, hematoxylin-eosin, original magnification × 200).

cal analysis revealed that the newly synthesized extracellular matrix in the polymer tissue contained cartilage-specific type II collagen.

After 50 days a mechanical consistency very similar to cartilage tissue was achieved. This *de novo* cartilage showed clusters of chondrocytes-like cells surrounded by a deposited matrix (Fig. 3).

DISCUSSION

In recent years knowledge has increased about the environmental conditions that favor chondrocyte growth and differentiation in tissue culture, leading to the implantation of chondrocytes and mesenchymal cells as a means of cartilage repair (7). Attempts to repair cartilage defects with isolated chondrocytes have failed because of the formation of fibrocartilage or hyaline cartilage of poor quality (2).

The experimental tissue culture technique described here offers a novel concept for the *in vitro* formation of vital cartilage transplants for reconstructive surgery. Autologous isolated human cartilage cells from tiny biopsies can be used to yield increased numbers of cells with conventional monolayer cell culture procedures (5, 6). Subsequently, the cells can be harvested, integrated, and anchored in a 3-D polymer carrier that provides a temporary artificial intercellular matrix to ensure tissue stability. After prolonged culture under constant conditions, collagen and proteoglycan molecules accumulate inside the cell-polymer tissue and aggregate to form appropriate matrix components, as is evident by the formation of an extensive fibril network visible with electron microscopy. A new biological matrix consisting mainly of newly synthesized collagen fibrils and proteoglycans gradually fills the internal spaces of the resorbable fleece, and thereby interconnects the chondrocytes and polymer fibers.

The fiber structure of the bioresorbable polymer provides a suitable carrier for the 3-D arrangement of the seeded isolated cartilage cells, and thus induces the *in vivo* formation of cartilage-like tissue (7). The fleece offers a maximum of available internal polymer surface and a minimum of solid polymer volume, and therefore allows sufficient cell attachment when coated with an appropriate adhesion factor (10).

In comparison with monolayer cell cultures, the tissue constructs described here contain a very high number of cells in a relatively small space. When cultured in conventional petri dishes, the culture medium must frequently be partially replaced, and there is increased risk of bacterial contamination. Our perfusion culture system provides an excellent alternative, as the cells need not be manipulated and conditions are kept constant during weeks in culture (8). The cells in the polymer tissue are separated from the moving culture medium by an agarose gel which mimics the in vivo situation, where nutrients from the blood stream can reach the cartilage cells only by diffusion.

In conclusion, our experiments demonstrate a promising pathway for the in vitro engineering of vital cartilage tissue suitable as autologous implantable material. New experiments are in progress with animal models in order to determine the *in vivo* behavior cartilage transplants prepared *in vitro*.

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