Engineering of cartilage tissue using bioresorbable polymer carriers in perfusion culture

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Bioresorbable polymer fleeces with a high internal surface area were used as temporary matrices to establish three-dimensional cultures of isolated human articular chondrocytes. The polymer surface was coated with poly-L-lysine to support cell attachment. The resulting cell–polymer tissues were cultured in perfusion culture chambers to achieve a constant supply of nutrients by diffusion. Retention and accumulation of extracellular matrix components synthesized by the chondrocytes were improved by encapsulation of the cell–polymer integrate in agarose gel. The cell–polymer tissues formed abundant collagen fibrils *in vitro* with a typical cross-triation clearly visible in electron microscopy analysis. Chondrocytes and intercellular matrix stained positively with monoclonal antibodies specific for differentiated chondrocytes and type II collagen. Synthesis of proteoglycans and collagen was also evident by further analysis with alcian blue and azan staining of cell–polymer tissue sections. The presented experimental tissue culture technique offers a novel concept for the *in vitro* formation of vital cartilage implants for reconstructive surgery or treatment of destructive joint diseases and possibly for the *in vitro* engineering of human tissues in general, with applications in drug testing and replacement of animal experiments.

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The regenerative capacity of cartilage is known to be very poor and is therefore a major problem in several diseases with severe cartilage loss. Defects of cartilage rarely, if ever, heal due to a lack of proliferative activity of the surrounding cartilage cells[1]. Attempts to repair cartilage defects using isolated chondrocytes have failed because of formation of fibrocartilage or hyaline cartilage of poor quality. In the present study a new approach is presented for the *in vitro* engineering of vital cartilage implants for autologous grafting to repair severe joint defects or to fill cartilage lesions in reconstructive surgery.

Cartilage tissue is characterized by chondrocytes and abundant extracellular matrix containing type II collagen and proteoglycans. When cartilage cells are isolated by enzymatic digestion and cultured as a monolayer they dedifferentiate, losing their rounded shape and acquiring a fibroblast-like appearance. Moreover, their collagen synthesis is switched from type II to type I[2,3]. However, when chondrocytes are cultured in agarose they have a more stable phenotype and such gel cultures are even able to induce redifferentiation of already dedifferentiated chondrocytes[4,5]. Agarose cultures have therefore opened interesting possibilities to promote the *in vitro* formation of cartilage tissue[6]. Cartilage cells can be amplified in cell number by serial subculture as a monolayer while losing their differentiated phenotype. Subsequently, this functional state can be regained upon transferring the cells into an appropriate carrier such as an agarose gel. This behaviour of chondrocytes should lead to procedures to prepare sufficient amounts of transplantable cells out of small autologous biopsies.

The major problem in the formation of an implantable vital cellular material is to find an appropriate delivery substance or cell carrier. Various attempts, mostly using gel-like substances, have been described so far[6,7]. Different approaches used resorbable sutures[8] or non-woven meshes[9] as carrier for chondrocytes. With gels like agarose, a sufficient homogeneous three-dimensional cell suspension can easily be achieved and cultured; however, these gel cultures lack the mechanical stability and behaviour required for implants in reconstructive surgery. Therefore, two prerequisites are necessary for the engineering of a vital implant. First, the three-dimensional cell carrier has to fulfil structural requirements; however, it has to

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be resorbed gradually subsequent to implantation. Second, a sufficient pre-formation and differentiation of a cartilage typic intercellular matrix has to be achieved *in vitro* prior to implantation in order to avoid the formation of fibrocartilage *in vivo*. Successful development of such vital implantable tissues would open broad possibilities for reconstructive surgery, treatment of destructive joint diseases and novel *in vitro* assays.

MATERIALS AND METHODS

Cell preparation

Hyaline cartilage was obtained from macroscopically normal femoral heads of humans between 30 and 65 yr of age within 12 h of death or from patients undergoing reconstructive surgery. Chondrocytes were isolated using an enzymatic solution, as described previously[10]. Briefly, the extracellular matrix of cartilage slices was digested for 12–18 h at 37°C in the presence of 2 mg/ml of type II collagenase (Seromed, Berlin, Germany), 0.1 mg/ml of hyaluronidase (Serva, Berlin, Germany) and 0.15 mg/ml of DNase II (Paesel, Frankfurt, Germany) in Ham's F12 medium. The resulting cell suspension was filtered through a nylon sieve with a pore size of 88 μm. Chondrocytes were suspended in Ham's F12 with 10% fetal calf serum (FCS; BRL, Eggenstein, Germany) in a spinner flask for 15 h. The resulting cell suspension was centrifuged at 1500 rpm for 7 min and resuspended in Ham's F12 containing 20% FCS.

Cell attachment assays

Analysis of attachment onto coated plastic dishes
Solutions of poly-L-lysine, fibronectin, chicken collagen type II (Seromed), and laminin and vitronectin (BRL) were applied as recommended by the manufacturers for coating wells of microtitre plates. Subsequently, equal numbers of chondrocytes were seeded into each well and were allowed to adhere for 20 min. Plates were then vibrated vigorously for 30 s. Thereafter, the medium and all unattached cells were removed. The relative amounts of bound cells were determined by MTT tetrazolium blue photometry detecting the cellular mitochondrial activity[11].

Analysis of attachment onto polymer meshes
Polymer meshes were dipped into solutions of adhesion factors for 30 min and subsequently lyophilized overnight. The meshes were seeded with a chondrocyte suspension and cells were allowed to attach for 20 min. Subsequently, the nets were washed and placed into microtitre wells with medium. The number of attached cells was again evaluated by MTT tetrazolium blue photometry.

Tissue engineering and culture

Bioresorbable co-polymer fleeces of vicryl and polydioxanon (Ethicon, Norderstedt, Germany) were soaked with poly-L-lysine or type II collagen and subsequently lyophilized. The prepared chondrocytes were loaded into the polymer fleeces by overlaying the biomaterial with the chondrocyte suspension in a tube. The biomaterials containing the cells were encapsulated with 4% low-melting agarose solution (Sigma, Deisenhofen, Germany). The pre-chilled agarose solidified within a few minutes by cooling it in cold water. The encapsulated cell–polymer tissues were placed into perfusion culture chambers (Minucells and Minutissue, Bad Abbach, Germany) and hooked up to a permanent flow of fresh medium[12]. In general, the pump cartridges were adjusted to deliver 1 ml/h of Ham's F12 culture medium (Seromed) supplemented with 2% FCS and ascorbic acid (50 μg/ml). The pump was operated with on/off intervals of 30 min throughout the entire culture period (*Figure 1*).

Histochemical analysis

The cultured tissues were harvested and cryopreserved in liquid nitrogen. Cryo sections of 15 μm were prepared for a panel of histochemical examinations. Monoclonal antibodies specific for differentiated chondrocytes and to collagens type I and type II (Chemicon, Temecula, CA) were used for APAAP immunohistochemistry to characterize cells and matrix synthesis. The markers for differentiated chondrocytes (Humc1, Humc3 and Humc4)[13], kindly provided by E. M. Lance, Shrinners Hospital for Crippled Children (Honolulu, HI), were negative in the vast majority of chondrocytes after they had been cultured in monolayer for 2 wk. Synthesis of proteoglycans was analysed by toluidine and alcian blue staining on fixed cryo sections of the cell–polymer tissue. Collagen synthesis was further investigated by azan staining.

Electron microscopy

For transmission electron microscopy (TEM), the specimens were fixed after perfusion culture for

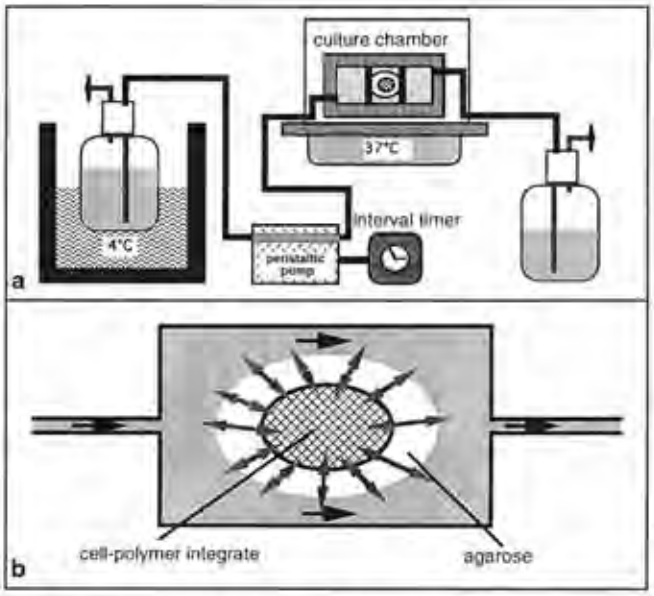

Figure 1 Perfusion culture. **a,** The interval timed peristaltic pump delivers 1 ml/h from a cooled reservoir through a completely closed culture chamber. **b,** An agarose coat separates the cell–polymer tissue from the medium flow.

20 min in medium containing 3% glutaraldehyde. Following 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. Semi-thin sections were stained with Richardson solution and examined under the light microscope. Ultra-thin sections were stained with uranyl acetate and lead citrate. The specimens were examined with a Zeiss electron microscope (EM 902).

RESULTS

Cell attachment

Using the culture procedures described, the chondrocytes rapidly adhered onto the bioresorbable carrier fleece. Various different attachment factors were tested for their capability to bind isolated cartilage cells. The numerous attachment assays revealed poly-L-lysine as the most efficient adhesion factor followed by type II collagen. Most chondrocytes were bound to culture wells coated with poly-L-lysine (data not illustrated). Polyglycolic acid (PGA) nets treated with poly-L-lysine also carried significantly more cells as determined by MTT than did meshes treated with collagen or untreated controls after they had been dipped into a chondrocyte suspension (*Figure 2*). The results of the MTT colour reaction were also confirmed by microscopic evaluation. A large amount of cells were seen to be attached onto the poly-L-lysine coated mesh, in contrast to hardly any cells on the control mesh. Out of the other adhesion factors examined, only collagen was found to significantly improve chondrocyte attachment compared with the control sample without an adhesion factor.

Tissue culture

Microscopic evaluation of cultured cell–polymer tissue specimens presented a rather homogeneous cell distribution depending on the homogeneous quality of the prepared fleece. Abundant intercellular matrix synthesis was apparent and in some cultures obviously filled most of the intercellular and inter-polymer-fibre space (*Figure 3*). A closer look at isolated groups or single fibres (12 μm in diameter) revealed numerous attached cells along and around the fibres' surface (*Figure 3*). Control fleeces without attachment factors contained much less cells, and the chondrocytes were distributed unevenly and preferably accumulated in disperse aggregates.

Matrix synthesis

The chondrocytes in cell–polymer cultures continued and maintained synthesis of collagen and proteoglycans as analysed by histochemical staining on cryo sections (*Figure 4*). Tissue sections presented an extensive matrix staining when treated with azan, alcian and toluidine. Positively stained zones were not only limited to the proximity of chondrocytes, instead they apparently even filled spans between more distant cells and fibres.

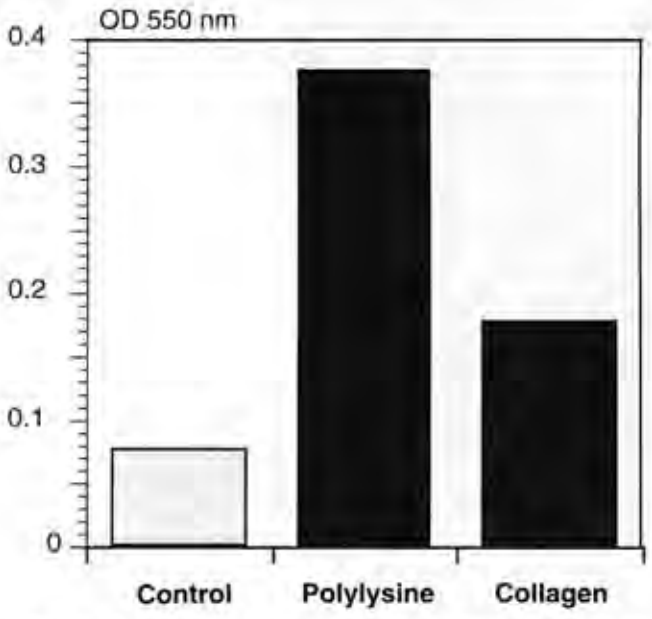

Figure 2 Attachment of chondrocytes to PGA meshes, as analysed by a modified MTT colorimetric assay.

Phenotype evaluation by immunohistochemistry

Immunohistochemical analysis revealed that newly synthesized extracellular matrix in the polymer tissue contained cartilage-specific type II collagen, as shown by monoclonal antibodies indicating a cartilage typic development of the new matrix. The cells, mostly presenting a rounded morphology, also stained clearly positive for two out of three chondrocyte-specific differentiation antigens beyond 14 days in culture. The third marker, however, was negative after only 1 wk of tissue culture.

Electron microscopy

Abundant collagen fibril formation was shown by electron microscopy (*Figure 5*). The collagen could be recognized by its typical periodical fibrillar staining pattern, which is usually not evident when chondrocytes are cultured as a monolayer.

DISCUSSION

The experimental design presented offers a novel concept of the synthesis and engineering of human tissues *in vitro* required as replacement material in reconstructive surgery. Thus, autologous isolated human cartilage cells from tiny biopsies can be amplified in cell number using conventional monolayer cell culture procedures. Subsequently, the cells can be harvested, and thereafter integrated and anchored into a three-dimensional polymer carrier, providing a temporary artificial intercellular matrix for sufficient tissue stability. With prolonged time in culture under constant culture conditions, collagen and proteoglycan molecules accumulate inside the cell–polymer tissue and aggregate to form appropriate matrix components, as evident by extensive fibril

Figure 3 **a**, Chondrocytes attached to fleece fibres coated with poly-L-lysine. **b**, Typical appearance of chondrocytes cultured in the polymer fleece.

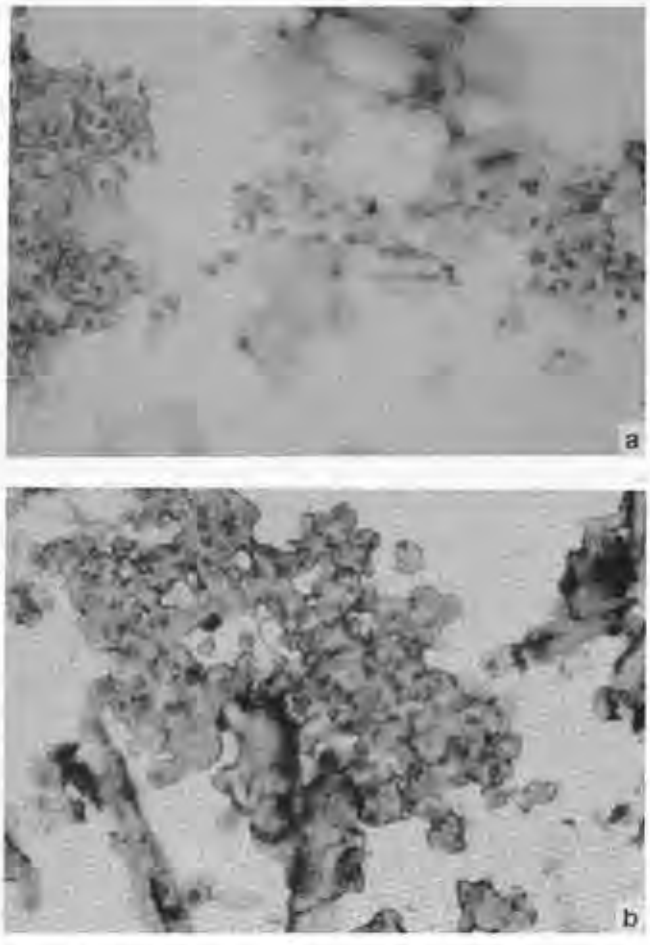

Figure 4 Histochemical analysis. **a**, Alcian blue staining on cryo sections. **b**, Immunohistochemical analysis of type II collagen synthesis.

formation visible by 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 is thereby inter-connecting the chondrocytes and polymer fibres.

It has long been known that chondrocytes present an unstable phenotype when cultured in a monolayer system. This dedifferentiation normally comprises a change of the cell shape from round cells to elongated cells with a fibroblast-like appearance and a switch in collagen synthesis from type II to type I collagen within 2 wk. Therefore, the success of *in vitro* formation of cartilage greatly depends on the maintenance of the phenotypic properties of the chondrocytes in the polymer tissue. First, the cell number has to be amplified in monolayer culture supported by growth factors such as epidermal and fibroblast growth factor[14]. Then, redifferentiation of chondrocytes in the cell–polymer tissue must be achieved, as already described for three-dimensional agarose cultures[4,15]. The cells have to regain their rounded morphology with the synthesis of mainly cartilage-specific type II collagen.

The fibre structure of the applied bioresorbable polymer provides a suitable carrier to achieve a three-dimensional arrangement of the seeded isolated cartilage cells. The fleece offers a maximum of accessible internal polymer surface and a minimum of solid polymer volume, and therefore allows sufficient cell attachment when coated with an appropriate adhesion factor. The fibre distances of the fleece as well as the polymer surface and the viscosity of the loaded single cell suspension are important parameters for a homogeneous distribution of cells within the tissue construct. During the first weeks in culture the polymer fleeces are robust and therefore very easy to handle if necessary; however, polymers based on polyglycolic acid usually strongly degrade after about 4 wk in culture, leading to a somewhat unstable tissue[16]. To overcome this problem, investigations have to be continued with a resorbable polymer that shows a considerably slower degradation process. Polymers of lactic acid offer degradation times of more than 1 yr[17] and therefore should allow long-term tissue development *in vitro*. Implants formed out of such polylactic acid tissues could secure sufficient fleece strength for an extended time after implantation until a newly synthesized extracellular matrix has matured to replace the polymer carrier in its function.

When compared with monolayer cell cultures, the newly described tissue constructs contain a very large number of cells condensed into a relatively small

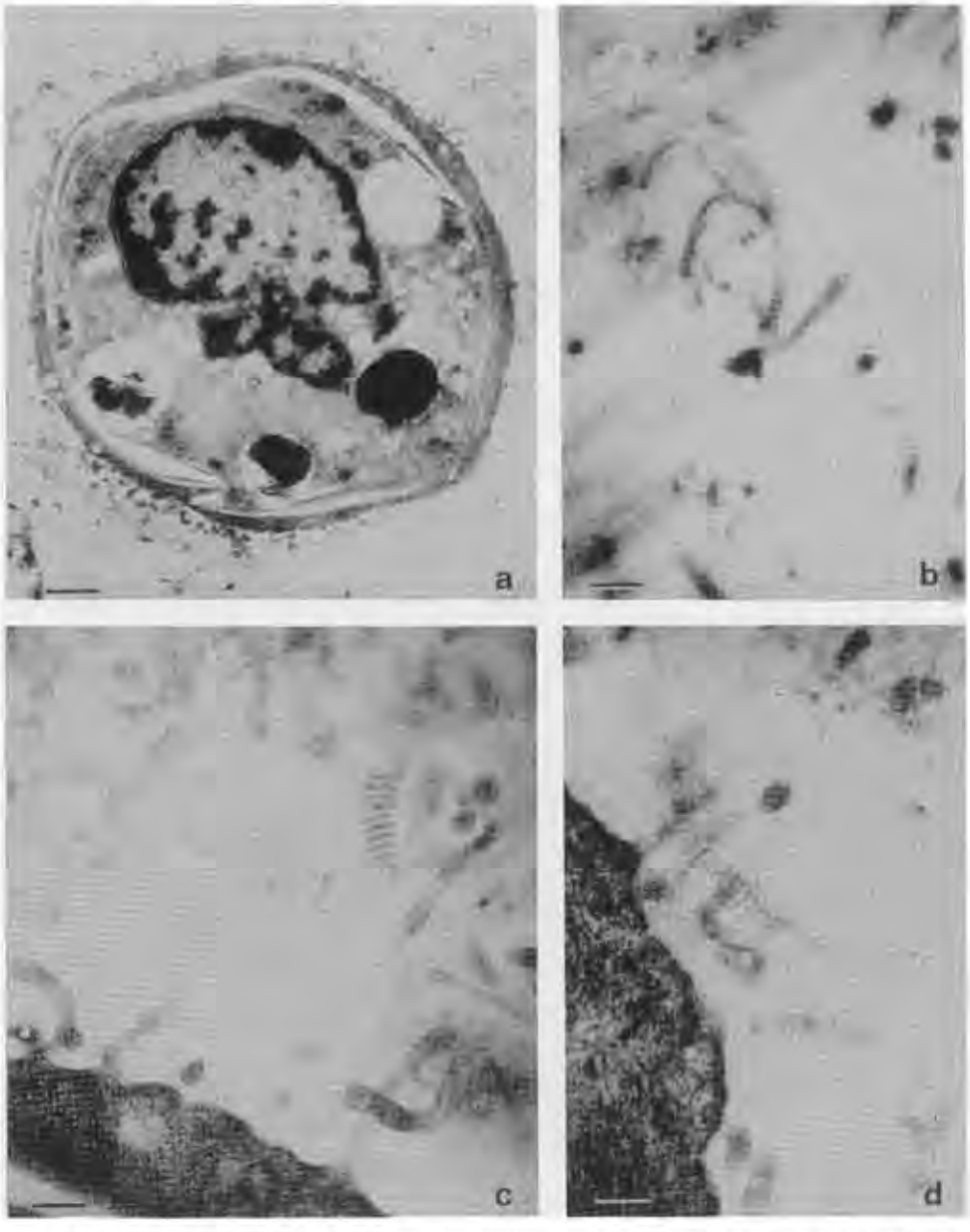

Figure 5 Transmission electron microscopy of chondrocytes cultured for 14 days after permanent perfusion of medium. **a,** Cross-section of a cultured chondrocyte. The plasma membrane shows numerous protrusions (bar = 1.1 μm). **b,** Within the lacunar regions near the chondrocytes newly synthesized collagenous networks are found in close contact with the chondrocyte protrusions. The collagen is recognized by its typical periodical staining (bar = 0.4 μm). **c** and **d,** Tangential sections of plasma membrane areas of cultured chondrocytes reveal numerous and long microvillar structures with an intensive synthesis of collagen fibres in the close vicinity (bar = 0.25 μm).

space. If cultured in conventional Petri dishes, a frequent and inconvenient partial renewal of culture medium is required. Moreover, the risk of bacterial contamination greatly increases. The applied perfusion culture system provides an excellent alternative, keeping cells untouched in constant conditions throughout weeks in culture. 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. The agarose further reduces the permeability for proteoglycans[18] and collagens, therefore accumulating synthesized matrix components in the intercellular space rather than allowing diffusion into the medium outside the tissue construct. Breakdown products, however, freely move out of the gel capsule. It is further known that ascorbic acid improves the retention of proteoglycans in chondrocyte cultures[19].

In conclusion, the experiments demonstrate a promising pathway for *in vitro* engineering of vital cartilage tissue suitable to prepare autologous implantable material. Furthermore, the development of such tissues could open broad possibilities to define *in vitro* model systems for human tissues. Improved assay systems for drug testing could be designed and animal experiments possibly replaced by more defined and differentiated tissues formed *in vitro*.

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REFERENCES

1 Campbell CJ. The healing of cartilage defects. *Clin Orthop* 1969; **64:** 45–63.
2 von der Mark K, Gauss V, von der Mark H, Muller P. Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. *Nature* 1977; **267:** 531–532.
3 Norby DP, Malemud CJ, Sokoloff L. Differences in the collagen types synthesized by lapine articular chondrocytes in spinner and monolayer culture. *Arthritis Rheum* 1977; **20:** 709–716.
4 Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell* 1982; **30:** 215–224.
5 Bujia J, Sittinger M, Pitzke P, Wilmes E, Hammer C. Synthesis of human cartilage using organotypic cell culture. ORL 1993; **55:** 347–351.
6 Solursh M. Formation of cartilage tissue *in vitro*. *J Cell Biochem* 1991; **45:** 258–260.
7 Robinson D, Halperin N, Nevo Z. Regenerating hyaline cartilage in articular defects of old chickens using implants of embryonal chick chondrocytes embedded in a new natural delivery substance. *Calcif Tissue Int* 1990; **46:** 246–253.
8 Vacanti CA, Langer R, Schloo B, Vacanti JP. Synthetic polymers seeded with chondrocytes provide a template for new cartilage formation. *Plast Reconstr Surg* 1991; **88:** 753–759.
9 Atala A, Vacanti JP, Peters CA, Mandell J, Retik AB, Freeman RM. Formation of urothelial structures *in vivo* from dissociated cells attached to biodegradable polymer scaffolds *in vitro*. *J Urol* 1992; **148:** 658–662.
10 Burmester GR, Menche D, Merryman P, Klein M, Winchester R. Application of monoclonal antibodies to the characterization of cells eluted from human articular cartilage: expression of Ia antigens in certain diseases and identification of an 85-kD cell surface molecule accumulated in the pericellular matrix. *Arthritis Rheum* 1983; **26:** 1187–95.
11 Miller RR, McDevitt CA. A quantitative microwell assay for chondrocyte cell adhesion. *Anal Biochem* 1991; **192:** 380–383.
12 Minuth WW, Rudolph U. A compatible support system of cell culture in biomedical research. *Cyto Technol* 1990; **4:** 1881–189.
13 Lance EM. Immunological reactivity towards chondrocytes in rat and man: relevance to autoimmune arthritis. *Immunol Lett* 1989; **21:** 63–73.
14 Kato Y, Hiraki Y, Inoue H, Kinoshita M, Yutani Y, Suzuki F. Differential and synergistic actions of somatomedin-like growth factors, fibroblast growth factor and epidermal growth factor in rabbit costal chondrocytes. *Eur J Biochem* 1983; **129:** 685–690.
15 Aulthouse AL, Beck M, Griffey E. *et al.* Expression of the human chondrocyte phenotype *in vitro*. *In Vitro Cell Dev Biol* 1989; **25:** 659–668.
16 Chu CC. An *in vitro* study of the effect of buffer on the degradation of poly(glycolic acid) sutures. *J Biomed Mater Res* 1981; **15:** 19–27.
17 Vert M, Li S, Garreau H. New insights on the degradation of bioresorbable polymeric devices based on lactic and glycolic acids. *Clin Mater* 1992; **10:** 3–8.
18 Verbruggen G, Veys EM, Wieme N, Malfait AM, Gijselbrecht L, Nimmegeers J, Almquist KF, Broddelez C. The synthesis and immobilisation of cartilage-specific proteoglycan by human chondrocytes in different concentrations of agarose. *Clin Exp Rheumatol* 1990; **8:** 371–378.
19 Jouis V, Bocquet J, Pujol JP, Brisset M, Loyau G. Effect of ascorbic acid on secreted proteoglycans from rabbit articular chondrocytes. *FEBS Lett* 1985; **186:** 233–240.