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Characteristics of human chondrocytes, osteoblasts and fibroblasts seeded onto a type I/III collagen sponge under different culture conditions

A light, scanning and transmission electron microscopy study

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Summary. Hyaline cartilage has only a limited capacity of regeneration, thus, lesions of articular cartilage can lead to early osteoarthrosis. Current concepts in conservative orthopedic therapy do not always lead to satisfying results. As one new attempt to facilitate cartilage repair, autologous transplantation of articular chondrocytes is investigated in different assays. This study was designed to create a resistible and stable cell-matrix-biocomposite with viable and biosynthetically active human chondrocytes, osteoblasts or fibroblasts. This biocomposite might serve as an implant to treat deep osteochondral defects in the knee. We collected cartilage, spongiosa and skin probes from healthy patients undergoing hip-surgery and enzymatically liberated the chondrocytes, seeded them into culture flasks and cultured them until confluent. The spongiosa and the skin samples were also placed in culture flasks and cells cultured until confluent. After 4–6 weeks, cells were trypsinized and grown on a type I/III collagen matrix (Chondrogide™, Geistlich Biomaterials, Wolhusen, Switzerland) for 7 days in standard Petri dishes and in a special perfusion chamber culture system. As controls, cells were seeded onto plastic surfaces. Then scaffolds were fixed and embedded for light microscopy and electron microscopy by routine methods.

Light microscopically, chondrocytes grown on the surface of the scaffold form clusters or a dense layer of sometimes rather fibroblast-like and sometimes roundish, chondrocyte-like cells. Only a few cells grow deeper into the matrix. In transmission electron microscopy, the cells have a rather chondrocyte-like morphology which emphasizes the matrix-induced redifferentiation after dedifferentiation of chondrocytes in monolayer-culture in culture flasks. Chondrocytes on plastic surfaces have a spinocellular aspect with little signs of differentiation. Grown on Chondrogide™, cells are more roundish and adhere firmly to the collagen fibrils of the scaffold.

Osteoblasts grown on the collagen scaffold and examined by light microscopy form a thin cell-layer on the surface of the matrix with a reticular layer of dendritic cells underneath this sheet. Transmisson electron micrographs show spinocellular and flat cells on the collagen fibrils. Scanning electron micrographs show large dendritic osteoblasts on plastic and a confluent layer of flattened, dendritic cells on the collagen scaffold.

Fibroblasts form a thick multi-layer of typical spinocellular cells on the collagen matrix. Fibroblasts grown on plastic surfaces and examined by scanning electron microscopy also show a dense layer of fibroblast-like cells.

For all three different types of cells no morphological differences could be seen when comparing cultivation in the perfusion culture system to cultivation in standard Petri dishes, although mechanical stress is believed to induce differentiation of chondrocytes.

Especially the observed partially differentiated chondrocyte-matrix biocomposite might serve as an implant to treat deep cartilage defects, whereas osteoblasts and fibroblasts seem to be less suited.

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Introduction

Human cartilage tissue has only a limited capacity for regeneration after damage. Cartilage is an avascular tissue with a low mitotic potential. Deep defects stay empty or result in a short-lived increase in metabolic products as one mechanism of self-repair. Mesenchymal elements from subchondral bone may also form new connective tissue. The repair tissue is often fibrocartilaginous and tends to deteriorate into fibrous tissue with poor mechanical stability. The persistence of lesions and the inability of the joint to withstand the demands of the mechanical environment may lead to early osteoarthrosis. Thus, the treatment of deep lesions of articular cartilage is a challenging problem in orthopedic therapy. Current concepts are subchondral penetration of the bone (Pridie 1959), the articular lavage and debridement of damaged cartilage (Jackson et al. 1988; Baumgaertner et al. 1990), microfracture techniques (Steadman et al. 1997) and abrasion arthroplasty (Johnson 1986). Other strategies are the transplantation of allogenic or autologous cartilage (Beaver et al. 1992; Bobic 1996) as well as osteotomy (Insall et al. 1984). All these therapies can help to diminish symptoms such as pain or joint swelling but cannot restore the original hyaline cartilage. Besides these, there have been recent attempts such as the tranplantation of autogenous periosteum (O'Driscoll and Salter 1986) and perichondrium (Homminga et al. 1990; Bruns and Behrens 1997) that can induce regeneration of hyaline cartilage and may also serve for larger cartilage defects, but with the risk of calcification and subsequent ossification of the graft (Bab et al. 1982). A new possibility is the autologous transplantation of chondrocytes. After enzymatically liberating the chondrocytes from their matrix they can be cultured in vitro (Klagsbrun 1979; Benya and Shaffer 1982) to be retransplanted into cartilaginous defects with promising results (Brittberg et al. 1994). The use of cell transplants requires a method of delivery and temporarily stabilizing the cells in the defect. For this reason an artificial matrix is, in part, necessary to allow ingrowth of cells, stimulate matrix formation and bind new cells and matrix to the host tissue (Paletta et al. 1992; Vacanti et al. 1994).

The aim of this study was to investigate the behaviour of different human cell types including chondrocytes, osteoblasts and fibroblasts seeded onto a type I/III collagen sponge and cultivated in a perfusion chamber system versus cultivation in standard Petri dishes. This mechanically stable cell-matrix biocomposite may be used for defect repair in articular cartilage.

Materials and methods

Cartilage, spongiosa and skin samples were collected from healthy 74- to 87-year-old patients with informed consent who underwent elective surgery for total hip joint replacement.

Cartilage was diced into 1–2 mm^3 pieces and placed into Falcon tubes containing 0.5% hyaluronidase (Sigma H3506) for 15 minutes at room temperature. After rinsing with PBS the pieces were washed three times with 0.25% trypsin (Biochrom KG L2133) and then incubated in 0.1% collagenase (Sigma C9891) and 0.25% trypsin for 20 minutes at 37 °C. To remove the matrix completely, pieces were then washed with PBS, transferred into a new Falcon tube containing Iscove's culture medium (Biochrom KG F0465) with 0.2% collagenase (Sigma C9891) and placed onto a shaker rotating at 200 rpm for 8 hours at 37 °C. The cell suspension was centrifuged and resuspended in Ham's F-12 culture medium (Biochrom KG FG 0815) containing 10% fetal calf serum (Biochrom KG S0115), 50 µl/ml penicillin/streptomycin (Biochrom KG A2213), 50 µl/ml glutamine (Biochrom KG K0282) and 50 µl/ml non-essential amino acids (Gibco BRL 11140-035). Cells were cultured until confluent in 80 cm^2 culture flasks covered with 0.1% gelatine in a humidified environment containing 5% CO_2 at 37 °C for 4–6 weeks.

Spongiosa was collected from the resected femoral heads, minced into pieces of approximately 5 mm^3, placed into Falcon tubes and washed several times with PBS. The spongiosa was then transferred into 80 cm^3 culture flasks containing DMEM culture medium (Biochrom KG T04110), 10% fetal calf serum (Biochrom KG S0115), 50 µl/ml penicillin/streptomycin (Biochrom KG A2213), 50 µl/ml glutamine (Biochrom KG K0282) and 50 µl/ml non-essential amino acids (Gibco BRL 11140-035). Cells growing out of the pieces of spongiosa were cultured until confluent for 3–4 weeks in a humidified environment with 5% CO_2 at 37 °C.

Skin samples were cut into pieces of approximately 3×3 mm, washed with PBS and attached to the bottom of 25 cm^2 culture flasks containing DMEM culture medium and additives as described above. Cells growing out of the skin were cultured until confluent in a humidified environment with 5% CO_2 at 37 °C for 3–4 weeks.

The different cell types were then trypsinized and counted. For better handling the type I/III collagen sponges (Chondrogide™, Geistlich Biomaterials, Switzerland) were placed into plastic setup rings of 13 mm diameter (Minusheet No. 1300) and seeded at a concentration of 2×10^5 chondrocytes/ml, 2×10^5 osteoblasts/ml or 3×10^5 fibroblasts/ml. One set of collagen sponges with cells was placed into a perfusion chamber system (Minusheet No. 1302) that permitted a constant flow of Ham's F-12 or DMEM culture medium containing the additives described above through the membranes at a rate of 1 ml/hour. The other set of collagen sponges with cells was placed into a 24-well plate, the culture medium being changed every three days. Unseeded membranes and cells grown on Thermanox™ plastic coverslips served as controls. Cells were incubated under these conditions for 7 days.

For light microscopy, specimens were fixed with Bouin's fixative, dehydrated through a graded ethanol series and stained with Mayer's hematoxylin-eosin and Masson-Goldner. For transmission electron microscopy, specimens were fixed with 2.5% glutaraldeyde in a 0.06 M sodium cacodylate buffer (pH 7.35) for 48 to 72 hours at 4 °C, rinsed in 0.2 M sodium cacodylate buffer and postfixed with 1% osmium tetroxide at room temperature. Samples were then rinsed in 2.4% sodium chloride solution, washed in 0.2 M sodium acetate buffer (pH 5.0) and block stained with 1% uranyl acetate in 0.2 M sodium acetate buffer (pH 5.0) in the dark for 30 minutes at room temperature. For dehydratation in alcohol and embedding in araldite, routine procedures were followed. Ultrathin sections (50–70 nm) were stained

with lead citrate (Reynolds 1963) and examined in a Philips 400 electron microscope. For scanning electron microscopy, cells were rinsed with warm PBS and fixed with 2% glutaraldehyde and 0.6% paraformaldehyde in a 0.06 M sodium cacodylate buffer for 24 hours at 4 °C. Samples were then dehydrated in graded series of acetone, dried in a critical-point dryer, sputter-coated with gold-paladium and examined in a Philips SEM 505 scanning electron microscope operated at 30 kV. Results were documented on APX 100 films (Agfa).

Results

This study shows that different types of human cells can be cultivated on the Chondrogide™ sponge under perfusion chamber conditions and also in standard Petri dishes. The type I/III collagen scaffold has two different sides. One side is rough and porous for better attachment of the cells seeded onto the scaffold, the other side is smooth and dense, facing the articular cavity and serving as an occlusive barrier for cells but not for fluids. The resorbable sponge is elastic, mechanically resistant and shows no shrinkage over longer periods of time.

Chondrocytes grown on plastic cover-slips have a spinocellular, fibroblast-like aspect with a round or ovoid nucleus (Fig. 1). Cells grown on the type I/III collagen scaffold form a multi-layered apical cell sheet with partially fibroblast-like, spinocellular cells (Figs. 2 and 3) and partially roundish, chondrocyte-like cells. Underneath the apical layer, cells invade the superficial areas of the matrix, forming a loose reticular cell sheet. Cells do not invade deeper into the matrix. Neither the handling of the cell-matrix biocomposite nor the procedures for fixating could destroy the scaffold or the cell sheet adhering firmly to the scaffold. The ultrastructure of most of the cells is chondrocyte-like rather than fibroblast-like, with an ovoid nucleus, extensive rough endoplasmatic reticulum and mitochondria (Fig. 4). Cells show close contact to the collagen fibrils of the scaffold and multiple areas with extrusion of granular material.

Scanning electron micrographs yield flattened and extensive cells on the plastic cover-slips with protrusions and microvilli on their surface (Fig. 5) whereas cells grown on the type I/III collagen matrix (Fig. 6) are round with microvilli on their surface, and processes wrapping around the collagen fibrils. Cultivation of cells in the above described perfusion chambers versus cultivation in standard Petri dishes did not result in different cell morphology, either in light microscopy or in scanning or transmission electron microscopy.

Osteoblasts grown on plastic cover-slips are flattened and dendritic with numerous processes (Fig. 7). Grown on the collagen scaffold, the osteoblasts form a thin, single layer of flattened cells (Fig. 8) on the surface of the matrix. Underneath the apical layer rather fibroblast-like and partly roundish cells assemble a network of cells with little invasion into the scaffold. Transmisson electron micrographs show spinocellular cells (Fig. 9) with a flat nucleus, prominent nucleolus, some rough ER and a few mitochondria. Like chondrocytes, the osteoblasts have close contact with the collagen fibrils of the Chondrogide™ scaffold. Grown on plastic cover-slips and examined by scanning electron microscopy (Fig. 10), osteoblasts have a dendritic and flat morphology, giving off various processes. Seeded onto the collagen matrix, cells adhere firmly to the matrix, have a flattened form and wrap pro-

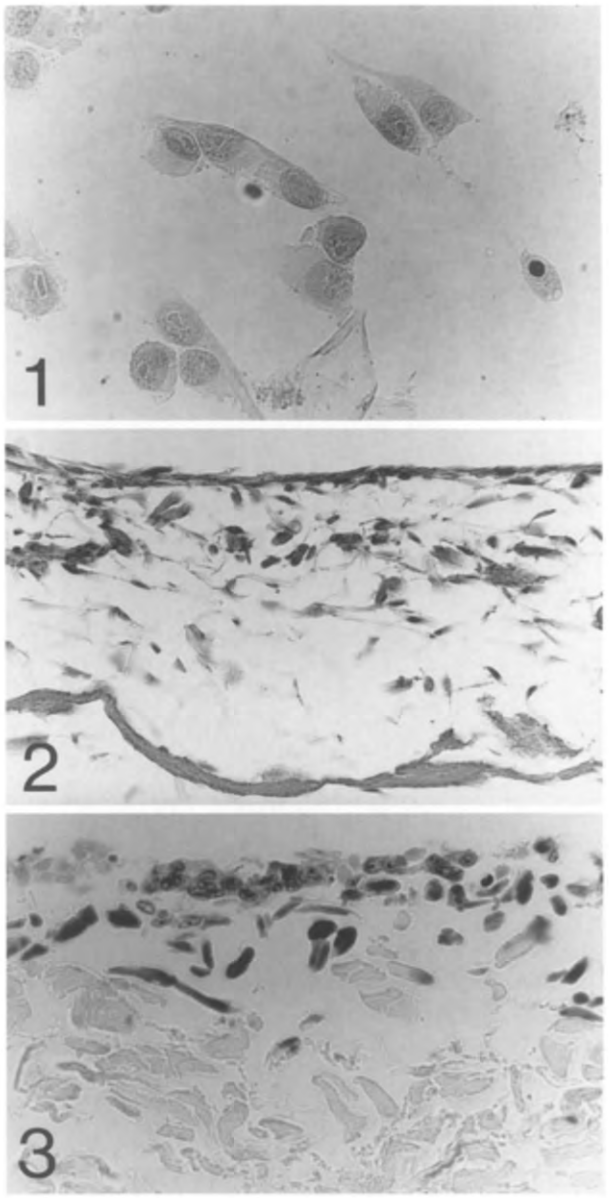
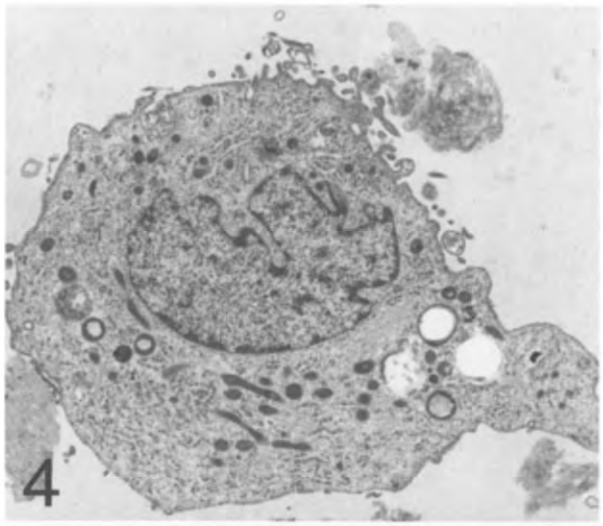
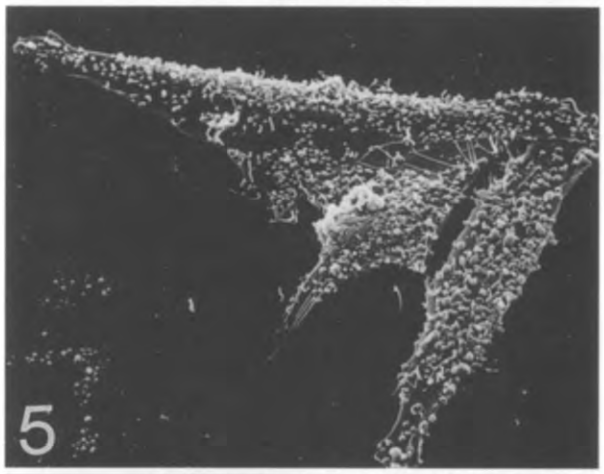


Fig. 1. Chondrocytes grown in monolayer on a plastic surface (Thermanox™). Light microscopy, HE-stained. The cells have a spinocellular, fibroblast-like aspect with round or ovoid nuclei. Magnif. ×220.

Figs. 2 and 3. Light microscopy of Masson-Goldner-stained chondrocytes grown on the Chondrogide™ scaffold. Cells form a multi-layered apical sheet with spinocellular aspect and a loose reticular sheet underneath. In other areas, cells form a dense multi-layer of roundish cells on the surface (arrow) not invading deeply into the matrix. Magnif. ×70, ×130.





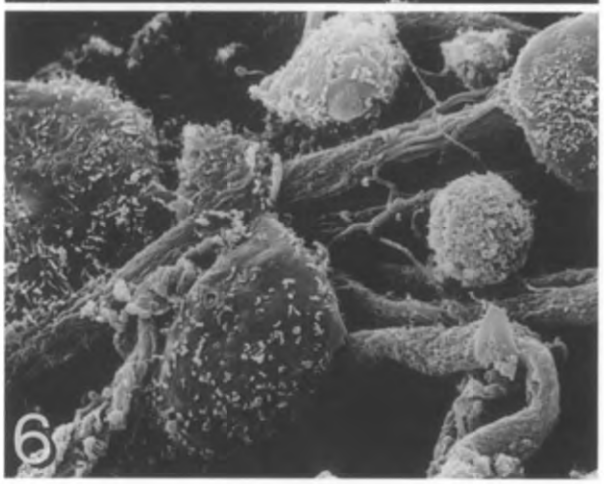


Fig. 4. Transmission electron microscopy of a chondrocyte grown on the Chondrogide™ scaffold. Note the irregular nucleus, the extensive rough ER and the mitochondria. Magnif. ×6000.
Fig. 5. Scanning electron micrograph of chondrocytes on a plastic surface with flattened and extensive cells. Note the protrusions and microvilli on the surface. Magnif. ×2500.
Fig. 6. Scanning electron micrograph of chondrocytes grown on the Chondrogride™ matrix. Cells are round with microvilli on their surface and wrap around the collagen fibrils. Magnif. ×2400.

cesses around the collagen fibrils (Fig. 11). Similar to the results above, no difference could be seen when comparing the perfusion culture system with the cultivation in standard Petri dishes.

Fibroblasts were grown on plastic cover slips as well, where they form a dense layer of typical spinocellular, fibroblast-like cells (Fig. 12) with ovoid nuclei. On the collagen scaffold they present a multilayer-sheet of flattened cells (Fig. 13) on the surface. Cells migrate into the sponge to the depth of about one quarter of its thickness. In transmission electron micrographs (Fig. 14) these cells have a spinocellular shape with a regular, ovoid nucleus, rough endoplasmatic reticulum, and a few mitochondria. They may sometimes contain lipid doplets and have close contact to the collagen fibrils of the scaffold. Scanning electron micrographs yield a dense layer of flat and spinocellular cells (Fig. 15) on the plastic cover-slips with close intercellular contacts and only a few vesicles on the surface. On the collagen scaffold, fibroblasts form a carpet-like layer of large cells exhibiting contacts with each other and adhering firmly to the matrix. They do not show microvilli or protrusions on their surfaces (Fig. 16).

Discussion

Focussing on the currently limited forms of treatment for articular cartilage defects which do not always lead to long-lasting and/or satisfying results, it is of great interest to develop cell-matrix constructs for transplantation to facilitate cartilage repair or even regeneration. In our study we therefore cultured human chondrocytes, osteoblasts and fibroblasts on a type I/III collagen scaffold under different culture conditions such as cultivation in a perfusion chamber (Sittinger et al. 1994) and cultivation in a standard Petri dish. The specimens were then examined by light microscopy, transmission and scanning electron microscopy.

Our results show that different types of human cells can be grown on a collagen scaffold using different methods of cultivation. When seeded with chondrocytes we can see the formation of a cell layer adhering firmly to the mechanically stable collagen scaffold. We observed areas on the scaffold where cells tended to redifferentiate into a rather chondrocyte-like morphology in contrast to the morphology of the cells cultivated on plastic. In other areas of the scaffold, cells had a rather undifferentiated and fibroblast-like morphology. Complete redifferentiation into chondrocyte morphology and biosynthetic activity has not been observed in our experiment.

Mechanical stress is believed to be essential for homeostasis and typical biosynthetic activity of chondrocytes (Buckwalter and Lane 1996; Lane Smith et al. 1995). Providing fluid-induced shear forces by the flow of culture medium through the scaffold in the biochamber system did not, however, yield differences in the morphology of the cells in our experiment. It is important to consider

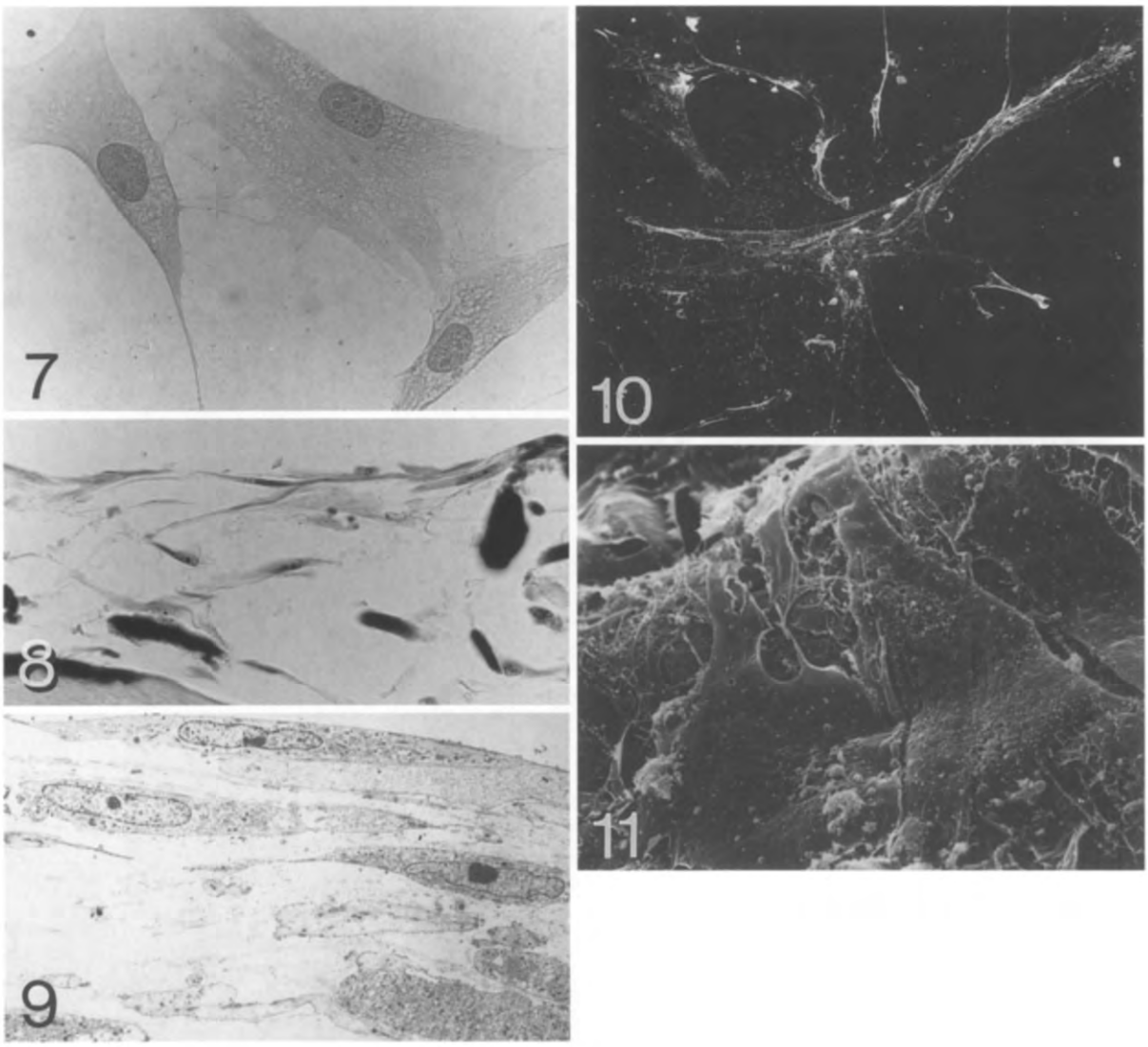
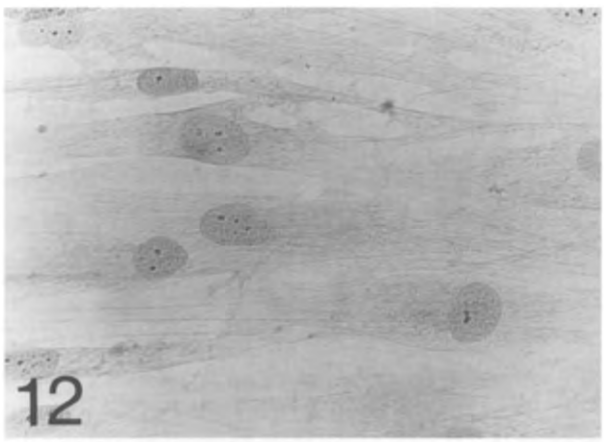
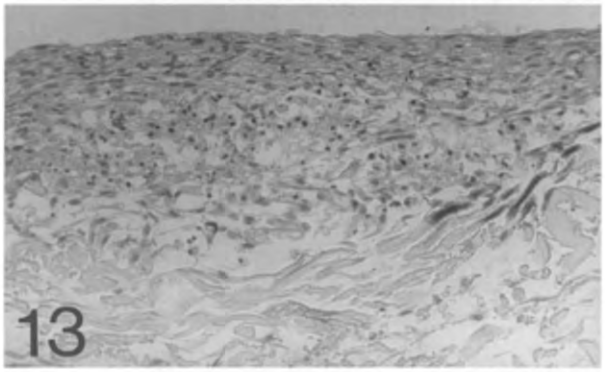


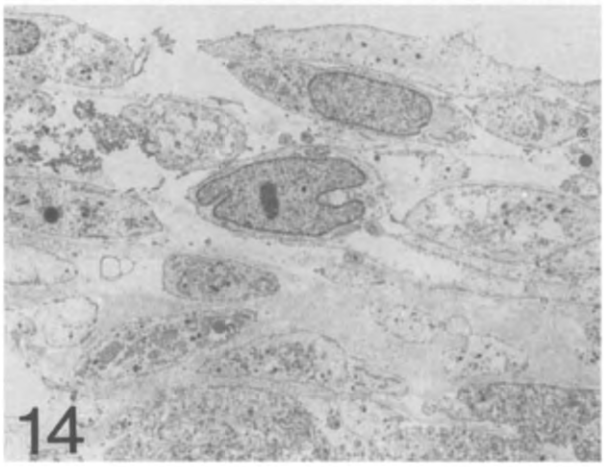
Fig. 7. Light microscopy of HE-stained osteoblasts grown in monolayer on a plastic surface (Thermanox™). The cells have a flattened and dendritic morphology with numerous processes. Magnif. ×220.
Fig. 8. Light microscopy of Masson-Goldner-stained osteoblasts grown on the collagen I/III scaffold with a thin apical layer of flattened cells and a network of cells with various processes underneath this layer. Magnif. ×70.
Fig. 9. Ultrastructure of osteoblasts grown on Chondrogide™. Cells have close contacts to the collagen fibrils and are of spinocellular aspect with flat nuclei, prominent nucleoli, rough ER and few mitochondria. Magnif. ×6 000.
Fig. 10. Scanning electron micrograph of osteoblasts grown in monolayer on a plastic surface (Thermanox™). Note the flattened and dendritic cells with spreading out processes. Magnif. ×2 500.
Fig. 11. Scanning electron micrograph of osteoblasts grown on Chondrogide™. Note the flattened cells adhering firmly to the matrix. Cells wrap processes around the fibrils of the membrane. Magnif. ×1 700.

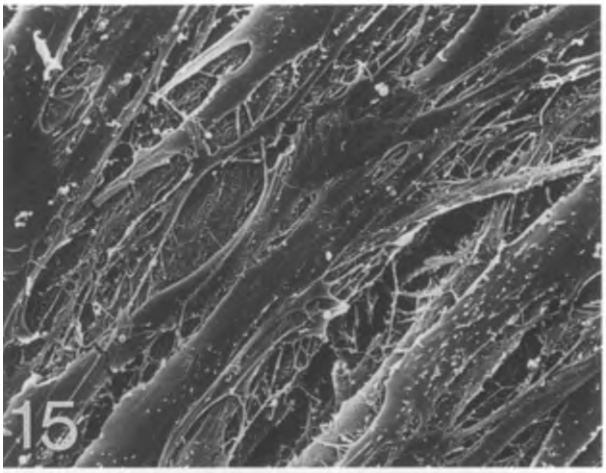
that the mechanical forces might have been of an unsuitable type or dimension, since these variables are not very fully described in the literature. Additionally, growth factors produced by the cells or important nutritional factors might have been washed away too rapidly to be utilized by the cells. But obviously, negative effects do not outweigh the positive effects of fluid-induced shear forces, so that even with growth factors and nutritional factors having been possibly washed away, cells do not tend to change their morphology in comparison with those cultivated in standard Petri dishes.

Chondrocytes are relatively difficult to collect, and their liberation out of the surrounding matrix and further handling is methodically demanding and time consuming. Since formation of cartilage by non-chondrogenic cell types might be possible (Nathanson et al. 1978) we also investigated other human cells seeded onto the collagen scaffold that are easier to collect and less difficult to handle. Periosteal grafts are known to have chondrogenic potential (O'Driscoll and Salter 1986). We investigated the potential of osteoblasts harvested from spongiosa for changing to a chondrogenic morphology when seeded









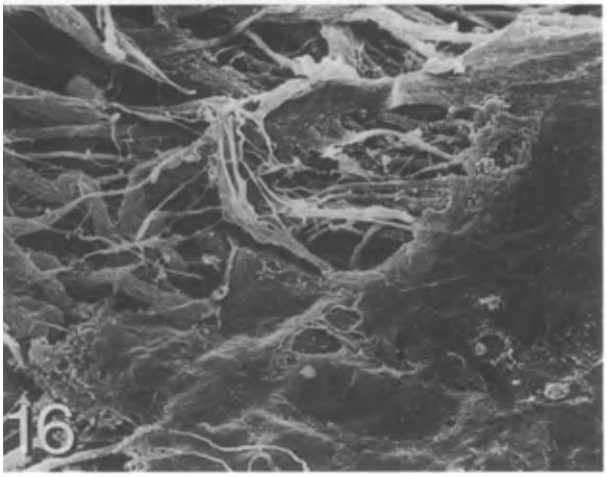


Fig. 16. Scanning electron micrographs of fibroblasts grown on Chondrogide™ forming a carpet-like layer of large, flattened cells adhering firmly to the matrix. Magnif. ×720.

Fig. 12. Fibroblasts grown in monolayer on a plastic surface (Thermanox™) with typical spinocellular aspect and ovoid nuclei. Magnif. ×220.

Fig. 13. Thick Masson-Goldner-stained layer of fibroblasts grown on Chondrogide™. Typical spinocellular and flat cells form a multi-layered sheet on the surface of the matrix. Magnif. ×40.

Fig. 14. Ultrastructure of fibroblasts grown on Chondrogide™. The cells show fibroblast-like shape with ovoid nuclei, rough ER and a few mitochondria. They may sometimes contain lipid doplets. Note the close contact to the collagen fibrils. Magnif. ×6000.

Fig. 15. Scanning electron micrograph of a dense layer of spinocellular fibroblasts grown on a plastic surface (Theramanox™) and a few vesicles in their surface. Magnif. ×2500.

onto a collagen sponge under special conditions of cultivation. Fibroblasts are able to induce the formation of cartilage under certain conditions such as cultivation on demineralized bone powder (Mizuno and Glowacki 1996) or with the use of certain factors like recombinant human bone-marrow-protein-2 (Hoshi et al. 1997). Our results show that osteoblasts and fibroblasts can also be cultivated on the collagen scaffold but do not show chondrocyte-like morphology under fluid-induced shear forces, fetal calf serum and the special architecture of the type I/III collagen sponge as factors generating chondroinduction of these cells. Osteoblasts as well as the fibroblasts show a rather dedifferentiated, spinocellular morphology. This indicates that the culture conditions

provided in our experiment are not sufficient to induce chondrogenesis of non-chondrogenic cells. Future studies will have to show whether the use of different factors like TGF-β, IGF, FGF-2 (Malemud 1993) or BMP's, different mechanical forces or new scaffolds can enhance the differentiation of non-chondrogenic cell types into chondrocytes. Additionally, one has to keep in mind that the typical matrix of cartilage mainly consists of type II collagen instead of types I and III collagen as in our matrix. Especially for chondrocytes and in part tor the osteoblasts and fibroblasts one would expect a higher grade of differentiation on a type II collagen matrix. In further experiments performed with a newly developed type II collagen matrix loaded with chondrocytes and cultured under standard conditions we could indeed see more roundish cells and production of proteoglycans than on the type I/III collagen matrix. Similar results are reported by Nehrer et al. (1997) with canine chondrocytes.

The question is, whether combining the newly developed type II collagen matrix seeded with chondrocytes under special culture conditions might lead to a higher grade of differentiation of the chondrocytes than we found on the type I/III collagen scaffold. Providing a matrix with special architecture made of the major constituent of natural cartilage, type II collagen, and additionally simulating mechanical stress and partially the pressure in a joint by fluid-induced shear forces in the perfusion chamber system, might be a promising way of generating cell-matrix biocomposites that are more differentiated. This highly differentiated cell-matrix construct could fulfill the functional capacities of cartilage more effectively. However, in biocomposites of high differentiation, problems might occur with less effective wound healing when implanted because the cells cannot migrate or divide, whereas less differentiated chondrocytes, like those we found on the type I/III scaffolds, are more motile and capable of dividing and thus might lead to a better fixation of the transplant. Moreover, less differentiated cells might divide further and expand the cell-matrix biocomposite in size, which is important for adapting the biocomposite exactly to the defect. Thus, the aim of future studies is to develop a transplant with differentiated chondrocytes and a cartilage-specific matrix possessing the functional capacities of cartilage but also containing cells that can still divide and migrate into the defect.

We believe that the type I/III collagen matrix-cell construct might lead to a matrix-induced semi-differentiation of still motile and mitotically active chondrocytes when transplanted into the defects, whereas osteoblasts and fibroblasts seem to be less suitable for this purpose.

The chondrocyte-matrix biocomposite is thus so far developed that it can be used for the repair of human knee cartilage defects, and this is currently being established in a clinical trial.

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