

Effect of Cytochalasin D on articular cartilage cell phenotype and shape in long-term organ culture

Pertti Pirttiniemi and Tuomo Kantomaa

Institute of Dentistry, University of Oulu, Finland

SUMMARY It has been documented, on the basis of cell culture experiments, that cytochalasin treatment promotes a round cell shape in chondroblast cultures by altering the cytoskeleton, and that it simultaneously alters the balance between production of type I and type II collagens. The aim in this study was to monitor the deposition of pro-type-I and type II collagens, and possible changes in articular cartilage layers in the mandibular condyle of the mouse under the influence of Cytochalasin D (CD) when total cranio-mandibular joints of 5-day-old mice were cultured in one block. The experimental group comprised 20 Balb/c mice of both sexes. Twenty *in vitro* controls were cultured without the administration of cytochalasin. The mice in the third group were used as *in vivo* controls.

The cells in the prechondroblast layer responded with a rapid change in shape when treated with CD and assumed a rounded morphology. The total thickness of the cell layer was reduced at 7 days. Immunostaining against pro-type-I collagen was intense in the narrow fibrous and prechondroblast layers in the CD-treated group, whereas the stained area was wider and the staining gradually reduced in the deeper cartilage layers in the *in vitro* controls. Staining against type II collagen became weaker at the end of the culturing period of the CD-treated group, whereas in the *in vitro* controls the staining against type II collagen was clearly visible at all observation times. These phenomena can be explained by changes in differentiation and the altered cell cycle of the chondroblasts in organ culture under the influence of CD.

Introduction

The differentiated functions of chondroblasts include synthesis, deposition and maintenance of the extracellular matrix. These components include mainly cartilage-specific collagens and proteoglycans. Type II collagen has been found to be specific to mature elastic cartilages, whereas type I is a product of immature and fibrous cartilage cells, and is present in the ossifying erosion layer of hypertrophic chondrocytes (Silbermann *et al.*, 1987; Strauss *et al.*, 1990; Schroter-Kermani *et al.*, 1991). Various cell culture models have provided valuable information on associations between the stability of chondroblastic phenotypes and factors affecting cartilage cell morphology (von der Mark *et al.*, 1977; Ben Ami *et al.*, 1991; Solursh, 1991; Sims *et al.*, 1992). The general effects of Cytochalasin D on cartilage

cultures carried out on a monolayer basis are well documented, and these experiments have helped to provide a general understanding of the span of the cartilage cell and its differentiation. A common finding in these experiments has been the dramatic dose-dependent effect of CD on cartilage cell shape, which is mediated by a local change in microfilament architecture as a consequence of partial depolymerization. The change is seen in a rounding of the cells and an onset of type II collagen production (Benya *et al.*, 1988; Newman and Watt, 1988; Aggeler, 1990). In spite of these findings, controversial views have been put forward concerning possible associations between the CD-induced alteration and the change in chondrocyte phenotype (Daniels and Solursh, 1991; Sims *et al.*, 1992). It has been speculated that cell rounding and the alterations in cell differentiation are separate phenomena, and

experimental evidence has also been obtained for this, as it has been possible to maintain specific expression of the cell culture, as seen in the type of collagen secreted, even when the cell configuration has been changed to represent an altered, dedifferentiated form (Benya, 1988). Increasing evidence based on monolayer cell-culture experiments has been accumulated to support the view that the organization of the actin cytoskeleton modulates the chondrogenic phenotype *in vitro* (Daniels and Solursh, 1991; Simpson *et al.*, 1993).

The purpose of the present investigation was to examine the deposition of pro-type-I and type II collagens, and possible changes in articular cartilage layers in the mandibular condyle of the mouse under the influence of Cytochalasin D when whole craniomandibular joints from 5-day-old mice were maintained in long-term organ culture.

Material and methods

Tissue preparation

Sixty Balb/c mice of both sexes were used. Forty of them were decapitated at the age of 5 days and the heads were swabbed with 70 per cent alcohol. The cranial base and the whole mandibles were dissected along the mid-sagittal plane into two blocks. Each explant was placed on its cut surface, the craniomandibular joint being positioned superiorly and supported by a fibre on a plastic culture dish containing Dulbecco's modified Eagle's medium (Gibco, Life Technologies Ltd., Paisley, Scotland) supplemented with 1 per cent sodium pyruvate (Gibco), 15 per cent fetal calf serum, 100 µg/ml streptomycin, 100 IU/ml penicillin and 15 mg/ml ascorbic acid. The cultures were placed in a humidified incubator at 37°C in a 5 per cent CO₂:95 per cent air atmosphere. The medium was changed every 48 hours.

The dishes were divided at random into two groups, those of the first group ($n = 20$) being maintained in the presence of 2 µg/ml Cytochalasin D (Sigma Chemicals, St Louis, Mo., USA). The controls ($n = 20$) were cultured without the administration of CD. Five explants from the experimental group and five from the controls were taken for fixation after culture

periods of 1, 3, 7, and 14 days, and the explants were embedded in paraffin without decalcification. Five of the most central sagittal sections of the condyles of 6 µm thickness were stained with toluidine blue and were used for histomorphometric measurements. The mice in the third group ($n = 20$) were used as *in vivo* controls and were killed at 6, 8, 12, and 19 days, analogously to the times when the organ culture specimens were taken for fixation.

The protocols were approved by the animal experimentation committee of the University of Oulu.

Immunohistochemical methods

The heads of the experimentals ($n = 20$), *in vitro* controls ($n = 20$) and *in vivo* controls ($n = 20$) were used for immunohistochemistry and histomorphometric analysis. The condyles were fixed in 10 per cent neutral formalin, demineralized for 10 days in 5 per cent formic acid, and embedded in paraffin. Three of the most central sagittal histological sections of the condyles of 6 µm thickness were analysed immunohistochemically with antibodies to type I procollagen and type II collagen. Sections for immunohistochemistry were digested for 1 hour at 37°C with 0.4 per cent pepsin. Pepsin (1.2 g, Sigma P-7000, 1780 U/mg) was dissolved in 288 ml of distilled water and 12 ml 0.25 N HCl were added. The sections were stained for type II collagen with a monoclonal antibody (CHD3) kindly provided by Dr Holmdahl (see Holmdahl *et al.*, 1986) and for type I collagen with an antibody to the carboxy-terminal propeptide of type I procollagen (PICP), an indicator of type I collagen synthesis activity, kindly provided by Dr Risteli (see Zhu *et al.*, 1993). The staining is able to demonstrate type I procollagen intracellularly, and also, to a lesser degree, extracellularly before it is converted to collagen extracellularly by specific enzymes and the carboxy-terminal propeptide is cleaved off (Melkko *et al.*, 1990). The antibodies were used at ×100 dilution and allowed to react overnight at 4°C. The reaction product was visualized with a Vectastain Elite kit (Vector Laboratories, Burlingame, CA) using a peroxidase and diaminobenzidine (Sigma D 5637) substrate. Negative

controls, using either PBS or non-immune rabbit IgG instead of primary antibody, were also prepared.

The sections used for type II collagen immunohistochemistry were counter-stained lightly with toluidine blue in order to better visualize the anatomical structure of the cartilage.

Measurement of cartilage layers

Five sagittal sections of 6 μm thickness at the centre of the joint were used for histomorphometry. The relative thicknesses of the three major cartilage layers were measured from the histological sections according to the classification of Luder *et al.* (1988), using light microscopy with an ocular grid. These measurements were performed on the most superior region of the condyle. A standardized sectioning technique was used when handling the specimens so that the orientation of the condyle with respect to the cutting surface and the glass, during staining, remained constant.

Digital image analysis

The differences in the profile of the chondroblast cell lacunae were examined by means of digital image analysis. Five sagittal sections of 6 μm thickness at the centre of the joint of the CD-treated mice and the *in vitro* controls were used for image

analysis. The sections were the same as those used for histomorphometry. The equipment comprised a digital image analyser (M1 Imaging Research Inc., Brock University, St Catharines, Ontario, Canada; Microscope, Nikon Optiphot II 40cx), the images being acquired using a CCD camera (Dage MTI 72E, Michigan City, Indiana, USA). A vertical line perpendicular to the superior articular surface in the centre of the condylar head was marked digitally. The morphology of each cell lacuna transversed by this line was analysed, its exact location and a profile factor indicating the ratio of the vertical to the transverse diameter being recorded. The method has been described in detail previously (Pirttiniemi and Kantomaa, 1996).

Statistical methods

One-way analysis of variance was used to determine the significances of the differences between the groups. The parametric test was used on the assumption that the sample was normally distributed.

Results

The thickness of the upper hypertrophic chondroblast layer was lower in the CD-treated group than in the *in vitro* controls after 1 day of culturing (Figure 1). After 7 days of culturing

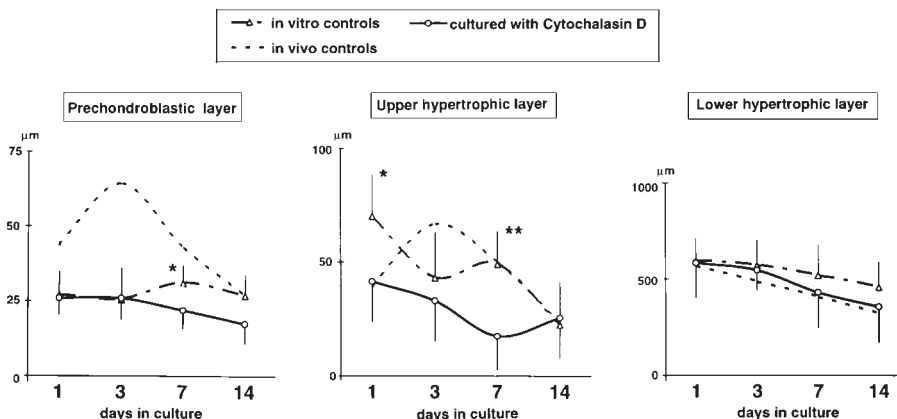
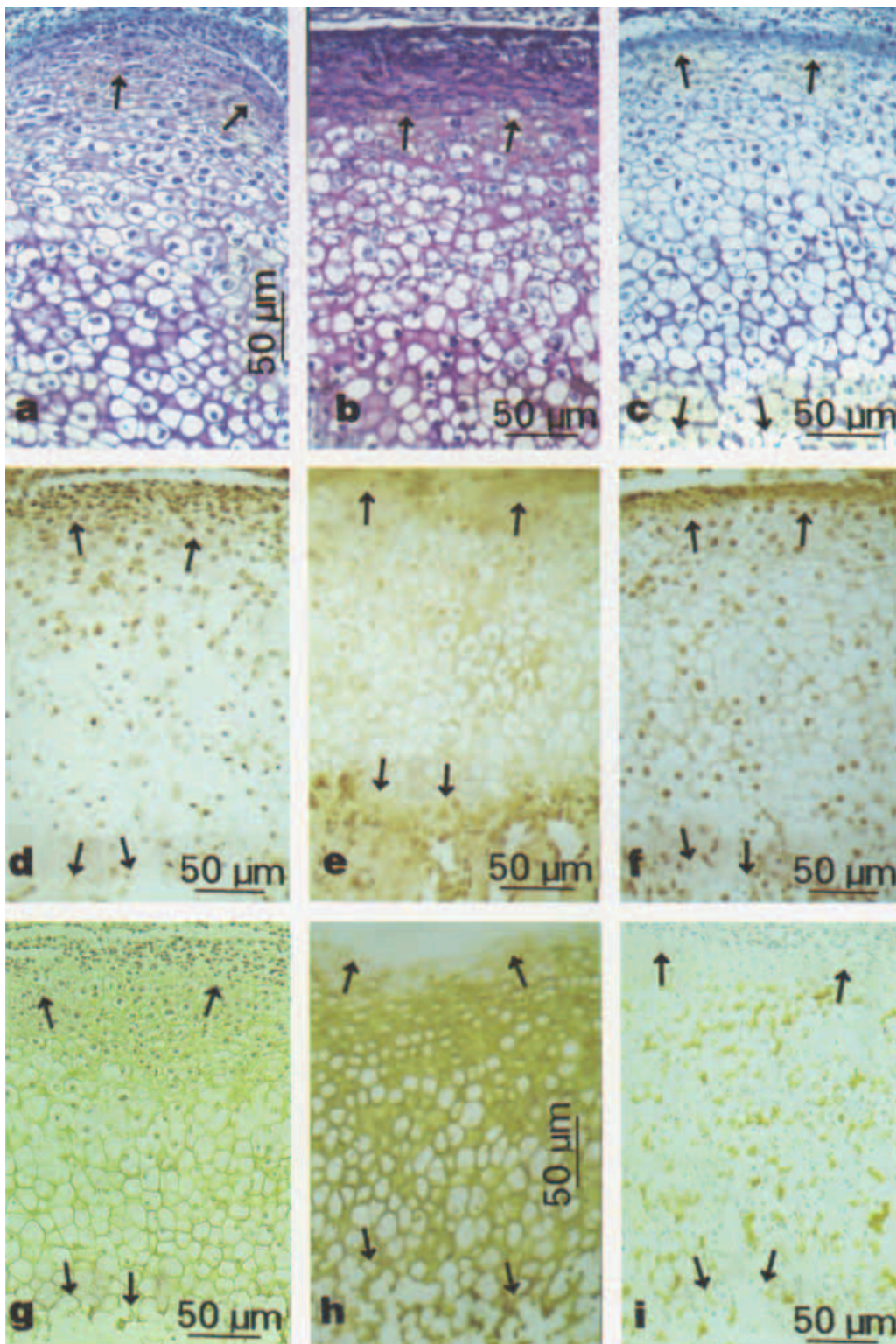


Figure 1 Thicknesses of three cartilage layers in μm (mean \pm SD) in the superior segment of the condyle of 5-day-old mice in the Cytochalasin D (CD)-treated group ($n = 20$) and two control groups ($n = 20$) after different culturing periods. The mean value for the *in vivo* controls (with no culturing) at the corresponding age is shown with a dotted line (SD not shown). The significant differences between the *in vitro* experimental and *in vitro* control groups (* $P < 0.05$, ** $P < 0.01$) are shown.



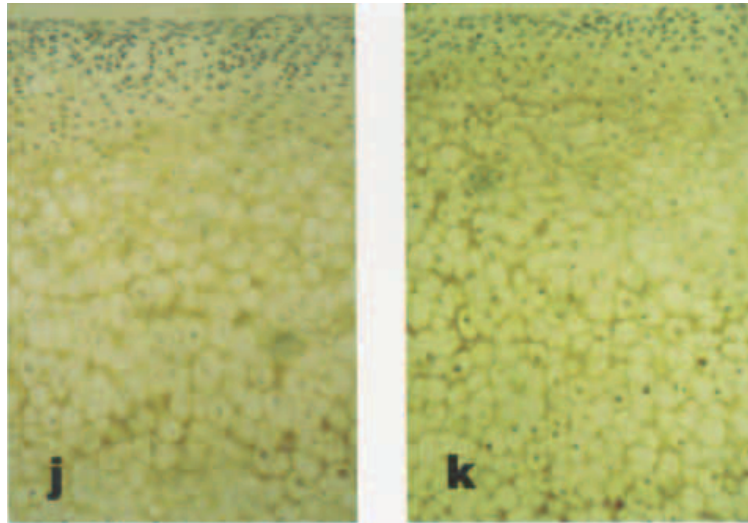


Figure 2 (a–c) Magnified views of mouse condylar cartilage layers after 7-day culturing. Toluidine blue staining. (a) In an *in vitro* control condyle the prechondroblast layer is narrow when compared with an *in vivo* control condyle (b). In a CD-treated condyle (c) the chondroblasts are rounded and the reduction of the extracellular matrix is clearly seen. The arrows show the superior border of the upper hypertrophic layer and the inferior border of the lower hypertrophic layer. (d–f) Magnified views of mouse condylar cartilage layers immunostained for type I procollagen (PICP), an indicator of type I collagen synthesis. No counterstaining was used. Fibroblastic and proliferative layers in a 7-day control culture (d) show intense intracellular and moderate extracellular immunostaining with decreasing intensity in the lower chondroblast layers. In an *in vivo* control mouse (e) the hypertrophied layers show considerably less staining than the prechondroblast layer. The immunostaining is increased in the mineralized tissue inferior to the erosion front. In a CD-treated condyle (f) the staining is mainly intracellular and the area of intensive staining is limited to a narrow prechondroblast layer. The arrows show the superior border of the upper hypertrophic layer and the inferior border of the lower hypertrophic layer. (g–i) Magnified views of mouse condylar cartilage immunostained for type II collagen and counterstained with haematoxylin. A 7-day *in vitro* control culture (g) shows consistent weak staining for type II collagen in the hypertrophic layers. In an *in vivo* control mouse (h), the immunostaining is intense in the upper and lower hypertrophic layers. Upon CD-treatment in a 7-day culture (i) there is no immunostaining for type II collagen in the proliferative layer, but the hypertrophic layer shows very weak extracellular staining. The arrows show the superior border of the upper hypertrophic layer and the inferior border of the lower hypertrophic layer. (j,k) A magnified view of mouse condylar cartilage immunostained for type II collagen and counterstained with haematoxylin (j). A 1-day *in vitro* control culture shows consistent staining for type II collagen in the hypertrophic layers (k).

the thicknesses of both the prechondroblast and the upper hypertrophic layers in the CD-treated group were smaller than those in the controls. There were no significant differences between the groups in the thickness of the lower hypertrophic layer (Figure 1).

The change in chondroblast morphology towards a round shape was visible after 1 day of culturing under the influence of CD, and this was most marked in the prechondroblast and upper hypertrophic layers, where the flattened chondroblasts, positioned parallel to the articular surface, gained a more spherical form. Simultaneously with the change in the shape of the chondroblasts, the proportion of extracellular

matrix decreased around the rounded chondroblasts, a change which was clearly visible after 7 days of culturing (Figure 2a–c).

Extracellular immunostaining against pro-type-I collagen was most intense in the narrow fibrous and prechondroblast layers, but was also seen in the hypertrophic layer extracellularly in the group treated with CD (Figure 2f), whereas the staining was gradually reduced in the deeper cartilage layers in the *in vitro* controls (Figure 2d). Both the organ cultured groups showed heavy intracellular staining against pro-type-I collagen in some cells of the hypertrophic layer, whereas in the *in vivo* controls the staining was mostly extracellular in the corresponding cell layers (Figure 2d–f).

Staining against type II collagen was lowered after 1 day culturing in the CD-treated group (Figure 2k) and became much weaker, and at the end of the culturing period when compared with both control groups (Figure 2g–i). In the *in vitro* controls (Figure 2j and g) the staining was visible in the upper and lower hypertrophic layers at all observation times, but the immunostaining was markedly weaker than in the *in vivo* controls (Figure 2h).

The behaviour of the profile factor for the chondroblast lacunae in the superior segment of the condyle is shown in Figure 3. The analysis was performed for the CD-treated group and for the *in vitro* controls, which showed a fluctuating, but relatively steady response curve in all cellular layers after treatment with Cytochalasin D, when the profile factor was correlated with the distance of the cell from the articular surface. However, in the control group a marked change from a flattened to a more ovoid cell profile was achieved in the deeper cellular regions of the condyle, a finding which is in line with the view in light microscopy (Figure 2a–f). The difference was significant at the distance of 100 μm from the articular surface ($P < 0.05$, analysis of variance).

Immunostaining for pro-type-I collagen and for type II collagen without the primary antibody are shown in Figure 4a and b, respectively.

Discussion

The present results confirm many of the findings recorded in cell culture studies. The response after administration of CD is equally as fast as far as cell shape factors are concerned. The rounding in the prechondroblast and upper hypertrophic layers is readily seen after 1 day of culture. This is an interesting finding as, in organ culture, where all the joint components are cultured in one block, pressure caused by the surrounding tissues could halt the cell rounding process. Earlier studies of monolayer cultures have shown an immediate response of the cartilage chondrocytes after the administration of CD in the form of changes in intra-cellular organization and in the expression of the chondroblasts, as seen in altered collagen secretion (Daniels and Solursh, 1991; Mallein-Gerin *et al.*, 1991; Simpson *et al.*,

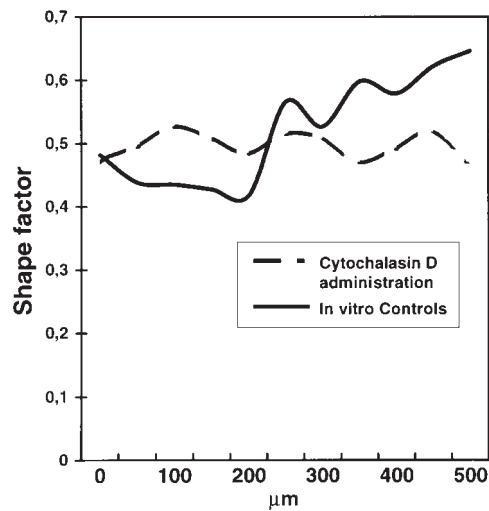


Figure 3 Cell profile factor, as related to the distance from the condylar surface (on x-axis) given by the image analyser for the chondroblast lacunae in the superior cartilage layers of the condyle of 5-day-old control ($n = 5$) and CD-treated mice ($n = 5$) cultured for 3 days. If the profile factor is one, the shape of the cell lacuna is spherical, while values below one indicate deviation from the round shape.

1993). When chondrocytes are maintained in cell culture under normal circumstances the cells often stop synthesizing cartilage-specific molecules such as type II collagen and cartilage proteoglycan and the secretion of type I collagen increases instead. This process has been called dedifferentiation, a term which does not correspond to the embryonic process (von der Mark *et al.*, 1977; Silbermann *et al.*, 1987; Watt and Dudhia, 1988). The effect of CDs on cultured chondrocytes has been shown to be dose-dependent, smaller doses having their effect on the secretion and expression of the cell, whereas larger doses also affect cell shape. The changes seen in cell shape have been shown to be determined by the structure of the actin cables. There is also strong evidence that microfilament modification may be a sufficient signal for the cells to change the type of collagen secreted (Benya, 1988; Benya *et al.*, 1988; Mallein-Gerin *et al.*, 1991).

When the CD-treated cartilage layers in the present experiment were observed, a marked reduction in extracellular matrix was seen in the

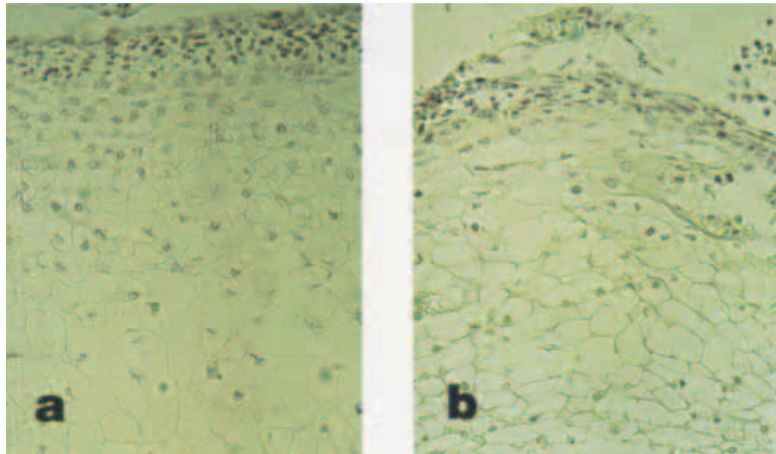


Figure 4 Negative controls using immunostaining for pro-type-I collagen without the primary antibody (a) and for type II collagen without the primary antibody (b). Counterstained with haematoxylin.

area of the rounded chondrocytes. This might be due to metalloproteinases, as one effect of cytochalasin treatment has been shown to be an increase in the secretion of stromelysin (Werb *et al.*, 1989; Aggeler, 1990; Simpson *et al.*, 1993), and stromelysin is a metalloproteinase which is able to break down proteoglycan—a major component of cartilage extracellular matrix. Analogously the decreased staining for type II collagen in the *in vitro* groups at the end of the experiment may at least partly be due to increased catabolism.

The rapid change in cell shape in the upper chondroblast layers is well in line with findings obtained with monolayer cultures. No such change was seen in the lower layers, however, which is probably related to the fact that the hypertrophied cells had already assumed a rounded morphology.

When condylar cartilage is cultured under different conditions, the general finding is a change in expression towards a 'chondroid' type, representing the dedifferentiated form with an increase in type I collagen expression, a decrease of type II collagen expression in the cartilage layers, and a change in cellular layer thicknesses simultaneously with an increase in hypertrophy (von der Mark *et al.*, 1977; Ben Ami *et al.*, 1991; Solursh, 1991; Sims *et al.*, 1992). A clearly

demarked thin area with extracellular type I procollagen was seen in the CD-treated group in this study after 1 week. In the *in vitro* controls, the area in the superior part of the condyle was much wider, pointing to the dedifferentiated nature of the chondroblasts, in agreement with the findings based on monolayer cultures. The hypertrophic layer in the experimental group showed a different response from that of the controls, as deposition of type II collagen was very weakly seen in the extracellular matrix after 1 week of CD-administration. This finding is divergent from the earlier cell culture experiments, as on monolayers an administration of CD causes an increased type II collagen expression (Benya, 1988; Benya *et al.*, 1988). The difference may be related to the fact that in organ culture with total joints the secretion of type II collagen continues, differing from the monolayer cultures (Silbermann *et al.*, 1987; Girdler, 1993). Cytochalasins have, on the other hand, an ability to halt the normal cell cycle to the S-phase (Simpson *et al.*, 1993), which phenomenon may, in long-term organ culture, reduce the number of cells able to secrete type II collagen and also be responsible for the reduction of the total amount of cartilage.

It is interesting that the image analysis finding showed a relatively unchanged profile curve in

all cellular layers after treatment with the Cytochalasin D, with more roundness of the cells in superior layers, but less roundness in the deeper layers than in the controls. The latter fact may be related to the possible decrease in the volume of the extracellular matrix which brings the cells in closer contact with each other thus forcing the cell lacunae to a polygonal shape, which phenomenon is seen in light microscopic sections.

Conclusions

Chondrocyte cell shape and the deposition of pro-type-I and type II collagens in organ culture are strongly affected by Cytochalasin D-treatment. We have demonstrated that some general principles found in cell culture studies can also be perceived in the organ culture model. The results differ especially in type II collagen deposition, which shows an adverse response, a finding which may be related to the different circumstances caused by the organ culture model. The use of total joints in organ culture, including articular cartilage, provides a useful model with which to examine these relationships.

Address for correspondence

Pertti Pirttiniemi
Department of Orthodontics and
Oral Development
Institute of Dentistry
University of Oulu
Aapistie 3
SF-90220 Oulu
Finland

Acknowledgements

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