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The in vitro influence of different culture conditions on the potential of sheep rib perichondrium to form hyaline-like cartilage

Evaluation of glueing materials used for in vivo graft fixation

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Abstract The effectiveness of autologous rib perichondrium for repair of full-thickness hyaline cartilage defects has been shown experimentally and clinically in various reports. The purpose of this study was to examine the behaviour of sheep rib perichondrial tissue under in vitro conditions and the influence of different culture matrices in order to evaluate possible stimulating effects. Rib perichondrium was obtained from sheep used for an experimental in vivo trial. After removal of adjacent cartilage remnants the tissue was devided and specimens cultured for 14 days in different ways. Explants cultured on collagen sponges (group A), fibrin glue (group B) and cellulose acetate filter (group C) were examined histologically, histochemically, histomorphometrically and autoradiographically. Clear differentiation of perichondrial cells towards a chondrocyte-like cell shape, particularly in the proliferation zone, was noticed on all matrices. These cells synthesized new matrix substances comparable to the ground substance normally present in hyaline cartilage. Morphometric comparison of tissue differentiation on different culture matrices revealed no significant differences in proliferation rates.

Key words Perichondrium · Cartilage defects
Tissue culture · Fibrin glue · Collagen sponge

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Introduction

The results of conservative or operative treatment of deep cartilagenous or osteochondral lesions are still unsatisfactory. The defects are replaced by scar tissue and osteoarthritis usually develops within a few years (Bentley 1978; Calandruccio and Gilmer 1962; Johnson 1986; Meachim and Roberts 1971; Mitchell and Shepard 1980; Nelson et al. 1988).

The chondrogenic potential of perichondrial tissue has been evaluated experimentally by several investigators demonstrating de novo chondrogenesis after autologous transplantation (Amiel et al. 1985a, b; Bruns et al. 1992a; Engkvist 1979; Engkvist and Ohlsen 1979; Engkvist et al. 1979; Homminga et al. 1989, 1990; Kon 1981; Skoog et al. 1972; Woo et al. 1987). Biochemical and biomechanical analysis of the newly formed hyaline-like cartilage revealed biological similarity to uninjured articular cartilage (Amiel et al. 1985a, b, 1988; Homminga et al. 1989; Maruyama 1979; Woo et al. 1987).

Because of these results perichondrial grafting has been used clinically for the repair of cartilage defects in different joints (Engkvist and Johansson 1980; Engkvist et al. 1979; Homminga et al. 1990; Niedermann et al. 1985; Pastacaldi and Engkvist 1979; Ritsilä et al. 1980, 1981; Serradge et al. 1984; Skoog et al. 1975; Tajima et al. 1978). To achieve good results application of continuous passive motion and restriction from weight-bearing is necessary for at least 3 months (Amiel et al. 1985a, b; Bruns et al. 1992a; Homminga et al. 1990). In order to shorten this period and to improve the quality of the newly developed hyaline-like cartilage further investigations evaluating facultative stimulating factors are necessary.

In principle, in vitro studies enable us to investigate such promoting agents without disturbing influences due to mechanical factors under in vivo conditions, but little is known about the in vitro behaviour of perichondrial tissue. Consequently, it was the first aim of this study to examine whether perichondrial tissue can be

cultured in order to establish an in vitro system for further evaluation of several potentially stimulating factors.

In addition, in vitro studies have suggested that added collagen structures promote differentiation of isolated cartilage precursor cells towards cartilage and/or bone cells (Kimura et al. 1984; Maor et al. 1987; Wakitani et al. 1989; Yasui et al. 1982) and clinical investigations (Gaudernak et al. 1986; Kaplonyi et al. 1988; Tiling 1986; Zilch 1986) have demonstrated the effectiveness of fibrin glue for refixation of osteochondral flakes in cases of trauma or osteochondritis dissecans. However, reliable factors which affect cartilagenous cell differentiation are unknown and a further aim of the study was to evaluate in vitro influences on perichondrial tissue following the additional application of either collagen sponges or fibrin glue.

Materials and methods

Perichondrial tissue was obtained from the ribs of five 5-month-old sheep, Awadassi/Ostfriesen strain, weighing 35–55 kg (from the Department of Animal Research Technion, Haifa). After removal of three explants per sheep, measuring 1.0 cm × 1.0 cm each, the tissue was immediately transferred to a vial containing saline (0.9% NaCl) and kept at 4° C. Within 1 h after removal the explanted tissue was carefully freed from adjacent cartilage remnants. Dissection of the explants was carried out using thin razor blades. The tissue was cut into pieces measuring 1.5 × 1.5 mm.

Perichondrial explants were placed in sterile petri dishes (Falcon, Oxnard, Calif., USA) containing 2 ml of culture medium (BGJ_b, modification of Fitton-Jackson; Bio. Ind. Beth HaEmek, Biomed. Prod. Div., Israel). Three to four pieces of perichondrial tissue were placed in each petri dish containing different culture matrices.

In group A specimens were cultured on collagen sponges (CS, product number M1332, diameter 16 mm, Helitrex, Am. Biomat. Corp., Plainsboro, N.J., USA) and in group B specimens were cultured on fibrin glue (FG; charge number P 36063407418611 T; Immuno, Heidelberg, Germany). In group C (control group) perichondrial tissue was cultured on cellulose acetate filter (pore size 0.45 µm, diameter 16 mm, Sartorius Goettingen, Germany).

These cultures were incubated in an incubator (Tuttnauer, Jerusalem, Israel) at 37° C in a humidified gas phase of 5% carbon dioxide in air. The culture medium was changed every second day.

In total, these perichondrial cultures including the three different experimental groups were repeated twice.

Specimens from each group were removed from the culture after 1, 3, 7, and 14 days and analysed histologically, histochemically, morphometrically and autoradiographically.

For histology all specimens were fixed for 5 days in 4% neutral buffered formalin and embedded in paraffin. Sections 6 µm thick were cut and stained with haematoxylin and eosin (H & E). Randomly chosen cuts of all sections were stained with toluidine blue at pH 2.0 and with periodic acid-Schiff reaction (PAS) at neutral pH for demonstration of acidic and neutral polysaccharides (Kiviranta et al. 1985; Silbermann and Frommer 1974; Silbermann et al. 1977).

Morphometrical analysis was performed using randomly chosen sections of all experimental groups, all days in culture and all three cultures and stained with H & E. The number of cells and isogenic cell groups (at least two nuclei/cluster) was counted in the proliferation and in the transition zone of the perichondrium (Fig. 1A) using a microscopic counting screen with an area of 0.04 mm^2.

For statistical analysis the results were analysed using Student's *t*-test.

For qualitative autoradiography, sections of each specimen, culture condition and day in culture were used. These sections were labelled with tritiated thymidine for the determination of the site of proliferation. Therefore, the culture medium was supplemented with 2 µCi/ml 24 h before the cultured tissue was harvested. The specimens were prepared for histological examination as described above. Sections were dipped in photographic emulsion (Kodak's emulsion NTB 2, Rochester, N.Y., USA), dried, and stored in the dark at 4° C for 3 weeks. The autoradiographs were developed in Kodak D-170 solution at 18° C, fixed in Kodak's rapid fixer, and washed.

Results

In total three series of cultures on each culture matrix (group A–C) were performed. Specimens of one culture (day 7, FG) had to be discharged because of microbial infection.

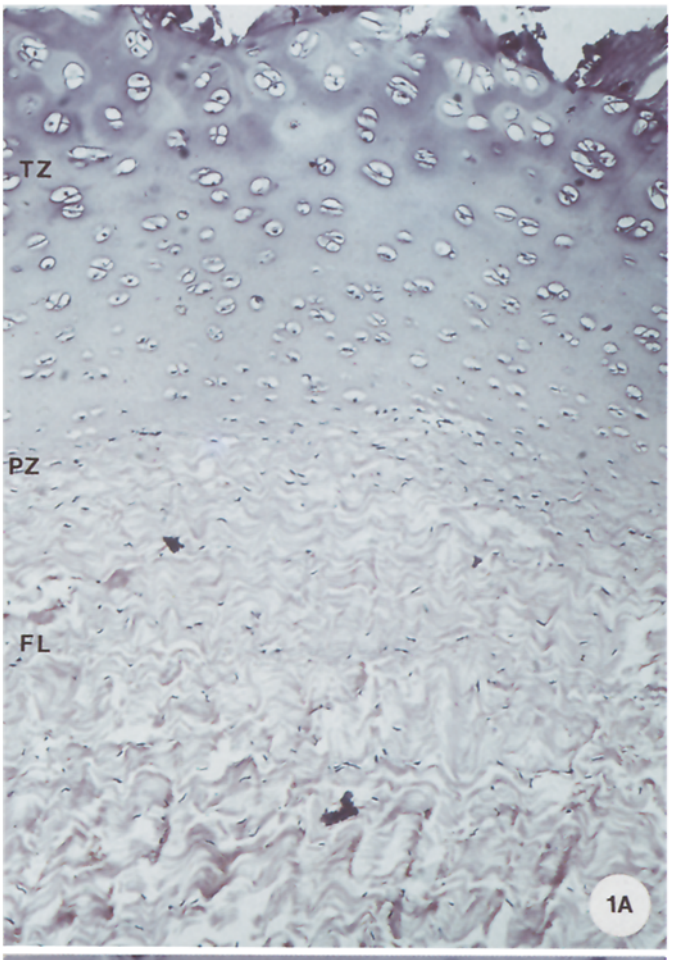
When perichondrium is harvested from the rib, there is a "natural" line of separation which can easily be found using a raspatorium. Histological examination of this tissue demonstrates three different layers (Fig. 1A). The outer layer, in situ the most peripheral part of the perichondrium, is called the fibrous layer (Bruns et al. 1992a, b; Engkvist et al. 1979) and consists of loose wave-like connective tissue with a small amount of fibrocyte-like cells. The intermediate layer, called the proliferation zone (Bruns et al. 1992a, b; Engkvist et al. 1979; Fig. 1A) contains elongated cells with oval nuclei thicker and less elongated than these of the fibrous part. This zone contains 3–5 rows of cells. The cells are surrounded by slightly stained ground substance. The inner layer (the transition zone; Bruns et al. 1992a, b; Engkvist et al. 1979) contains cells with a more rounded cell shape, rounded nuclei and large areas of cytoplasm. Cells are arranged in groups of at least two cells (Fig. 1A). Histochemical analysis with toluidine blue demonstrates no staining of the fibrous layer and the proliferation zone, whereas in the transition zone a slight staining with increasing intensity towards the rib cartilage is visible.

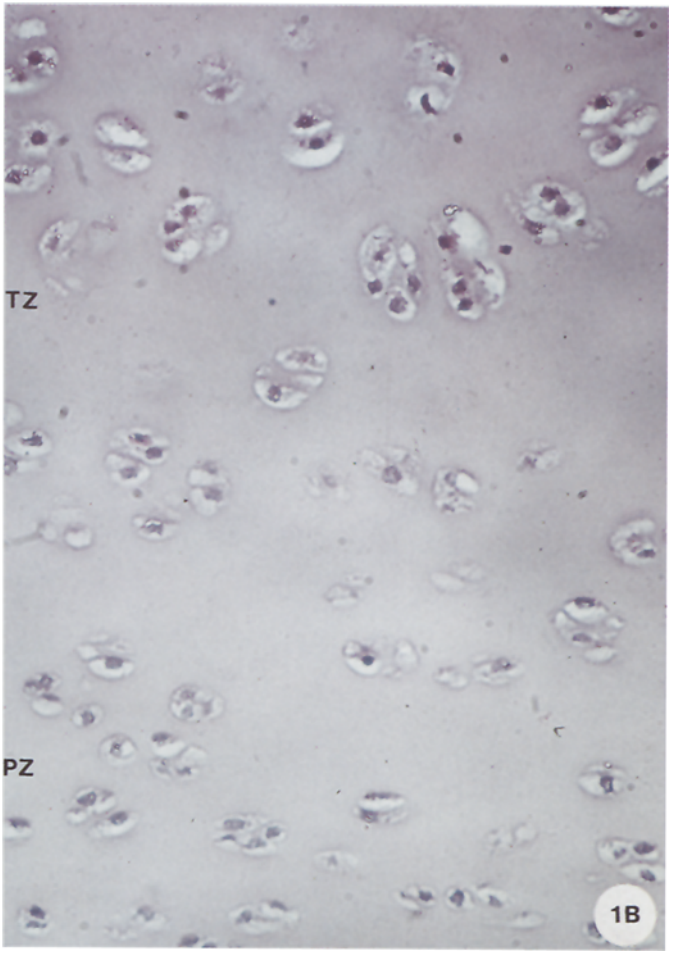
In contrast with that, slight staining with PAS in the fibrous layer, moderate staining in the proliferation zone and intense staining in the transition zone towards the rib cartilage is detectable.

Histological and histochemical analysis of all specimens after 1 day in culture demonstrates no difference between the three culture conditions and to the original non-cultured tissue (Fig. 1A).

After 3 days in culture all specimens of group C (control group) showed no histological change compared with the original tissue. The fibrous layer seems to be looser. In all specimens from group A and B more rounded nuclei were observed in the proliferation zone. Histochemically, specimens of all groups (A–C) show a slightly positive toluidine blue stain in the ground substance around the cells of the proliferation zone.

The PAS reaction, indicating production of neutral polysaccharides and intracellular production of glycogen, shows a slightly increased intensity in the ground





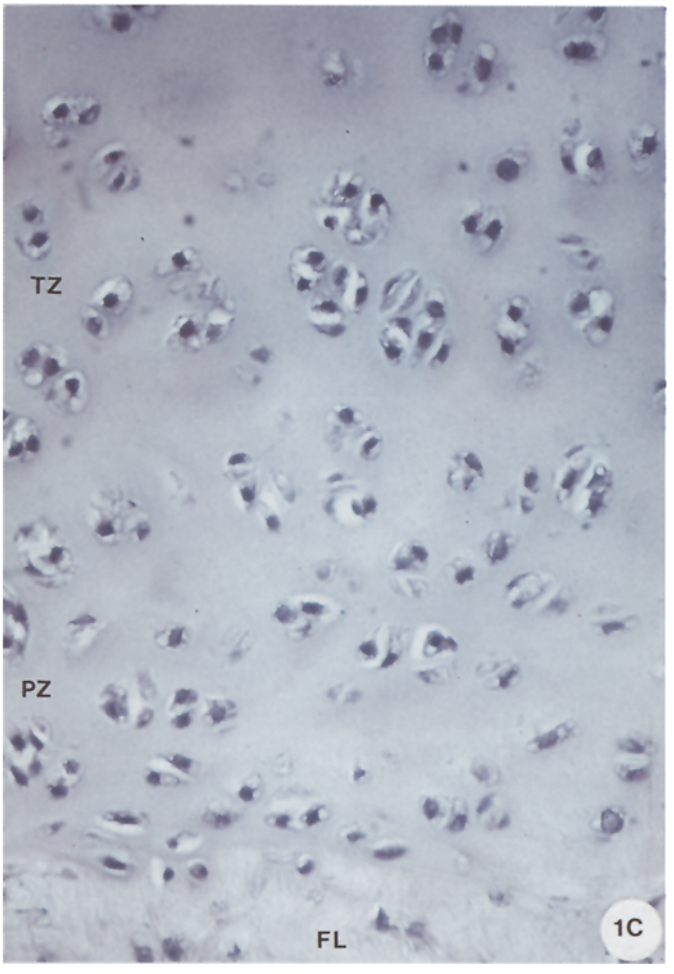


Fig. 1 **A** Periochondrial tissue after 1 day in culture on cellulose acetate filter (CF). The specimen demonstrates three layer: the outer layer, called fibrous layer (*FL*), the thin intermediate proliferation zone (*PZ*) and the inner layer adjacent to the rib cartilage, called transition zone (*TZ*). The *PZ* contains thin elongated cells with spindle-shaped nuclei. Cells in the *TZ* are more rounded, nuclei are surrounded by a large area of cytoplasm. Haematoxylin and eosin (H & E), × 40. **B** Specimen after 7 days in culture on fibrin glue (FG). Cells in the *PZ* are now more rounded, lie in groups of at least two cells per chondron and contain rounded, intensely stained nuclei. There is a less difference when compared to the *TZ* than in specimens after 1 day in culture H & E, × 64. **C** Specimen after 14 days in culture on FG. Only a slight difference between cells or chondrons in the PZ and TZ is visible. Most of the cells in the PZ lay in groups of at least two cells per chondron and show rounded, intensely stained nuclei surrounded by a large area of cytoplasm. In comparison to specimens after 7 days in culture (Fig. 1B) there is only a slight difference between the PZ and TZ. At the bottom the FL is visible. H & E, × 64

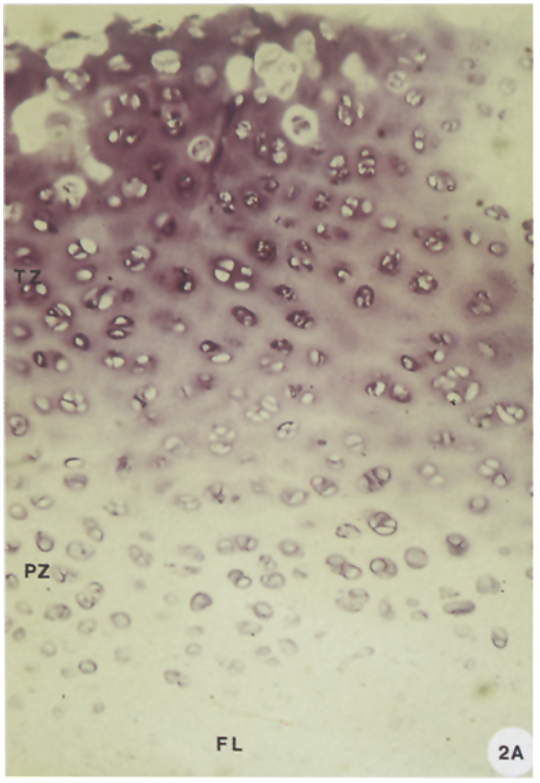
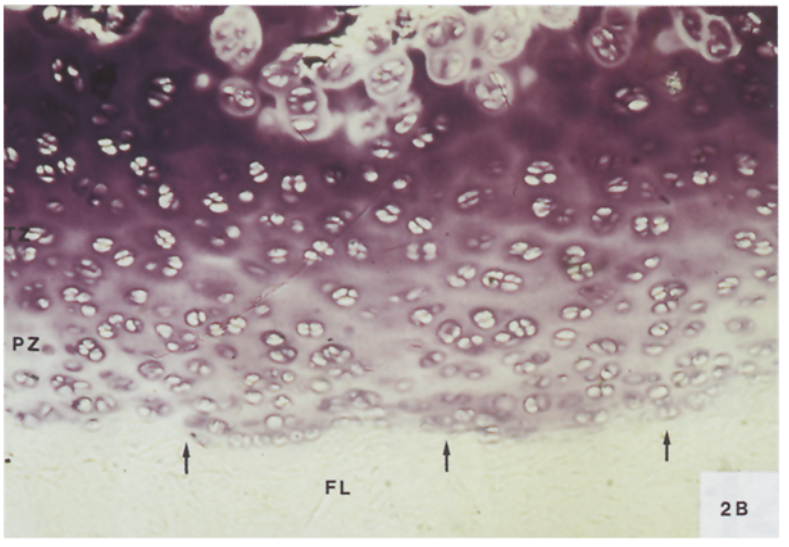


Fig. 2 **A** Histochemical stain of a specimen cultured for 7 days on collagen sponge (CS). The FL is not stained; the PZ demonstrates only a slight pericellular stain. In contrast, the ground substance of the TZ shows intense staining. Toluidine blue (pH 2.0), × 80. **B** Specimen after 14 days in culture on CF. A distinct border is visible between the PZ and the FL (*arrows*). The ground substance is intensely stained with an increase towards the TZ. Toluidine blue (pH 2.0), × 48



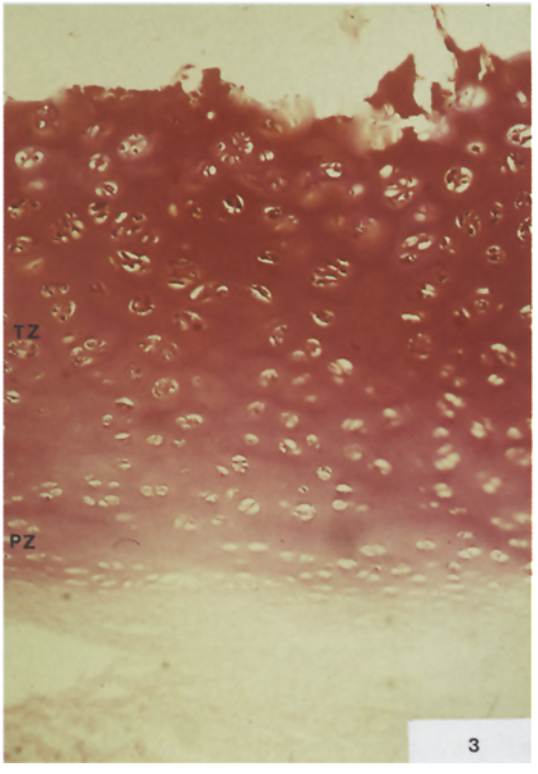
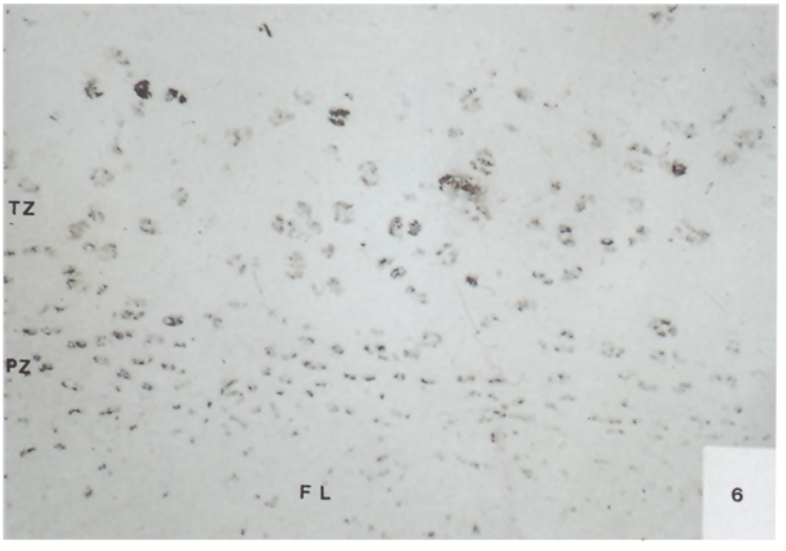


Fig. 3 Specimen after 14 days in culture on CF. Intense staining is visible in the PZ and TZ, indicating production of neutral polysaccharides. Additionally, cells of the TZ demonstrate intense intracellular staining indicating the production of glycogen, in contrast with cells of the PZ. Periodic acid-Schiff, × 48

Fig. 6 Autoradiography of a specimen after 3 days in culture on CF. Intense staining, indicating cell proliferation, is visible in the PZ and in the TZ. Cells in the FL show little radioactivity. Tritiated thymidine, H & E, × 40



substance and the cells of the proliferation and transition zone compared with the original tissue.

After 1 week in culture all specimens of the three groups (A–C) demonstrate a marked increase of the cell density and a distinct change of the cellular shape but no obvious differences between the groups. The former arrangement in layers (transition zone, proliferation zone) is diminished. Cells in the proliferation zone are now more rounded with large areas of translucent cytoplasm and large intensive stained rounded nuclei. Cells in the proliferation zone are now arranged more in groups of at least two cells (Fig. 1B). Histochemically, a

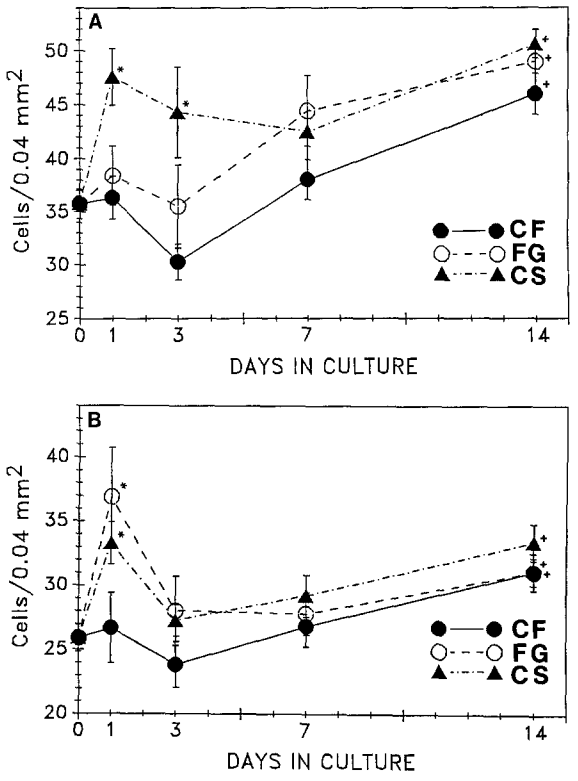


Fig. 4 A Graph demonstrating the cell density in the PZ depending on the different culture matrices. **B** Graph demonstrating the cell density in the TZ depending on the different culture matrices. Significant difference when compared with specimens at the same time interval; + significant difference in comparison to non-cultured perichondrium

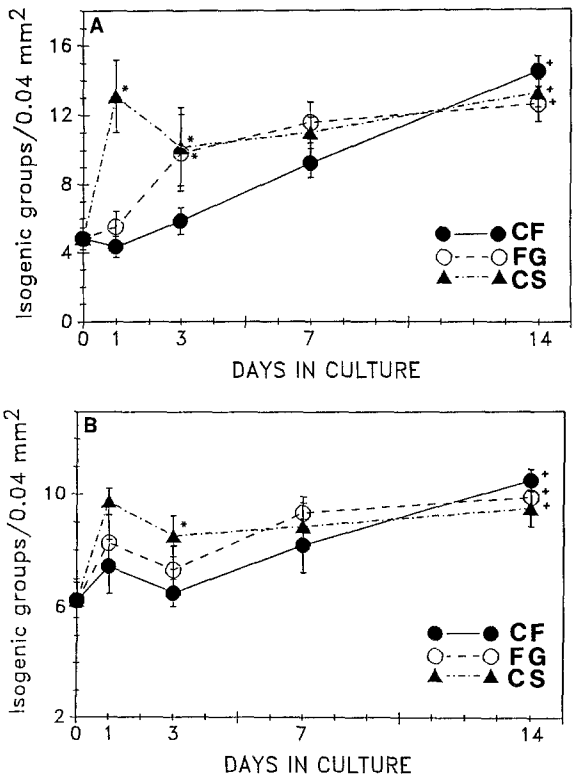


Fig. 5 A Graph demonstrating the number of isogenic groups in the PZ depending on the different culture matrices. **B** Graph demonstrating the number of isogenic groups in the TZ depending on different culture matrices. * Significant difference when compared with specimens at the same time intervall; + signifikant difference in comparison to non-cultured perichondrium

more intense staining with toluidine blue is visible in the proliferation zone with a marked pericellular halo (Fig. 2A).

After 14 days in culture the proliferation zone and the transition zone of specimens of all groups (A–C) demonstrate a further differentiation towards hyaline-like cartilage. Cells of both zones are now even more rounded; there is an obvious increase in the cell density and in the number of cells per chondron. All cells of the proliferation and transition zones demonstrate a chondrocyte-like shape (Fig. 1C). Histochemically, toluidine blue staining demonstrates a distinct border between the proliferation zone and the fibrous layer in all specimens of groups A–C. In the proliferation zone an intensely stained ground substance is visible; whereas, in the fibrous layer no staining is detectable (Fig. 2B). PAS reaction shows less distinct differences when compared with specimens after 7 days in culture, but an increase of intracellular staining is visible in the transition zone (Fig. 3). In contrast, intracellular staining is less intense in cells of the proliferation zone. Comparison of specimens from groups A–C reveals no obvious histological or histochemical difference.

Morphometrically, a slight increase of cell density and density of isogenic cell groups is seen in the transition zone and a distinct increase of cell density and density of isogenic groups in the proliferation zone is present. These changes are statistically significant (Figs. 4A, B, 5A, B).

Different culture matrices reveal a significant higher cell density of specimens cultured on FG and CS on morphometrical analysis in comparison with specimens of group C after 1 (and 3) days in culture (Figs. 4A, B, 5A, B).

Exemplary autoradiographical examination demonstrates a maximum of radioactivity in the proliferation zone and an intensive activity in the transition zone of specimens after 1 and 3 days in culture. No obvious differences between groups A–C (Fig. 6) were visible.

Discussion

Since Tizzoni (in 1878) and Doerner (in 1905) mentioned the chondrogenic potential of perichondrium, several experimental and clinical investigations have confirmed this characteristic (see Bruns et al. 1992a). However, few in vitro studies using perichondrial tissue or perichondrial cells have been performed. Gluecksmann, in 1939, was the first to report on the in vitro development of hyaline-like cartilage derived from perichondrium of metatarsals and phalanges of chick embryos and Engkvist et al. (1979), reported on in vitro experiments with isolated perichondrial tissue from rab-

bit ears. Development of hyaline-like cartilage was clearly demonstrated but the rate of microbial infection was high. Upton et al. (1981) cultured isolated cells derived from perichondrium of the rabbit ear after collagenase digestion and found that even isolated perichondrocytes were able to produce hyaline-like cartilage. More recently, Bulstra et al. (1990) corroborated the in vitro capacity for human rib perichondrium to form hyaline-like cartilage, but neither stimulating agents nor the glueing materials used clinically for graft fixation were examined. The background for the use of our particular culture substrates is that FG is commonly used for in vivo graft fixation and CS is known to have a stimulating effect on the cartilaginous and osseous cells differentiation from several in vitro investigations (Kimura et al. 1984; Maor et al. 1987; Wakitani et al. 1989; Yasui et al. 1982).

Our results from group C (control group) confirmed the typical course of cell differentiation towards a hyaline-like cell configuration. In contrast to Bulstra et al. (1990) we suggest the proliferation zone of the perichondrium to be the most active area for this production. This is also supported by the autoradiographic results where at least equal radioactivity was found for the proliferation and transition zone. However, for transplantation purposes this is of minor interest because perichondrial tissue as transplanted contains the proliferation zone, the transition zone and the fibrous layer. Furthermore, a suggested promoting effect on cartilaginous cell differentiation from the use of CS was not observed. There was no greater cell differentiation and no increased production of ground-substance when compared with a "standard" in vitro condition (group C).

The presumed promoting effect (Kimura et al. 1984; Maor et al. 1987; Wakitani et al. 1989; Yasui et al. 1982) was probably not detectable because cartilage precursor cells of the perichondrium were already surrounded by extracellular matrix which may hinder influences of the CS on the perichondrial precursor cells. Additionally, ground substance itself is known to promote cartilaginous differentiation of cartilage precursor cells (Carrino et al. 1983; Kosher et al. 1973; Nevo and Dorfman 1972; Schwartz and Dorfman 1975; Solursh and Meier 1974) and may have reduced the influence of the CS. Cartilage precursor cells in the perichondrium are arranged three-dimensionally, which is known to have a promoting effect on cartilage cell differentiation (Wakitani et al. 1989; Yasui et al. 1982), and there may be an additional effect due to the groundsubstance surrounding the perichondrocytes. Probably, this surround diminishes the influence of the partial pressure of oxygen on the perichondrocytes, resulting in maintainance of the hyaline-like differentiation.

Regarding the use of xenologous FG as culture matrix (group B) there was neither evidence of degenerative effects as suggested by Itay et al. (1987) nor of any stimulating influences on the development of hyaline-like cartilage. Comparison of results from group A (CS) and group B (FG) demonstrated no significant differences in the proliferation capacity of perichondrium. From these in vitro data no preference for the use of either FG or CS as a glueing agent can be given.

In conclusion, this study corroborates clearly the potential of rib perichondrium to form hyaline-like cartilage in vitro. In contrast, no promoting effects due to the use of FG or CS could be detected using morphological criteria.

Since several in vivo studies have demonstrated advantages of perichondrial grafting in the treatment of articular cartilage defects it is of particular clinical interest to examine factors which can possibly promote cartilaginous differentiation from perichondrial tissue. If any promoting effect due to the use of hormones, vitamins (Kato et al. 1984), other biochemicals agents or biophysical conditions such as low oxygen tension (Nevo et al. 1972), electromagnetic fields (Sakai et al. 1991) or intermittent compressive forces (Veldhuijzen et al. 1979) is detectable in vitro, this might be used as an intra-articular adjuvant in order to shorten the post-operative period when continuous passive motion and restriction from weight-bearing is necessary. It may also improve the biochemical and biomechanical quality of the newly formed tissue.

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