Optimization of chondrocyte expansion in culture

Effect of TGFß-2, bFGF and L-ascorbic acid on bovine articular chondrocytes

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In vitro multiplication of isolated chondrocytes is needed to repair articular cartilage defects with autologous material. In this study we used monolayer cultures of bovine articular chondrocytes. The effect of transforming growth factor ß-2, basic fibroblast growth factor or L-ascorbic acid on cell multiplication, in the presence of 10% fetal calf serum, was measured in primary culture, the third and tenth passage. TGFß-2 stimulated the proliferation of chondrocytes in the primary culture and L-ascorbic acid stimulated in the third passage. On the basis of these results, we chose an optimal addition scheme in which TGFß-2 was added in primary culture and first passage, followed by addition of L-ascorbic acid in the second and third passage; this resulted in a 7-fold increase in cell number compared to the control group, in about 4 weeks. Our findings stress the importance of adding the right growth factor at the right moment.

Collagen type II expression was lost after the third passage, in the control as well as in the experimental condition. The ability to produce hyaline cartilage specific matrix components is essential, if multiplied cells are to be used to repair cartilage defects.

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The use of cultured chondrocytes to repair articular cartilage defects has been considered successful (Brittberg et al. 1994, Noguchi et al. 1994, Kawamura et al. 1998). Repair of full-thickness articular cartilage defects has the best chance for success if the defect is isolated, without degenerative changes in the joint. Since defects of this kind are mainly found in young adults, experiments with young adult articular chondrocytes have clinical relevance. Allografts may lead to immunological rejection of the graft and transmission of infectious diseases; this makes autografts preferable. The number of chondrocytes that can be harvested from a patient is limited. Therefore the cells need to be multiplied in vitro. Several research groups have studied the effects of growth factors on this multiplication process. Chondrocytes dedifferentiate during multiplication in monolayer (Benya and Shaffer 1982). This can alter the response of chondrocytes to growth factors (Galéra et al. 1992, Guerne et al. 1994).

We used articular chondrocytes from 12–18-month-old bovine steers, which can be considered as young adults. Transforming growth factor ß-2 (TGFß-2), basic fibroblast growth factor (bFGF) and L-ascorbic acid are all able to stimulate chondrocyte proliferation under certain conditions (Adolphe et al. 1984, Blanco et al. 1995, Venezian et al. 1998). These factors were added to the culture medium in the presence of 10% Fetal Calf Serum. We studied the effects of the 3 factors on chondrocyte multiplication in primary culture (differentiated chondrocytes), in the third passage (partly dedifferentiated chondrocytes) and in the tenth passage (dedifferentiated chondrocytes). Furthermore, a scheme for sequential addition of growth factors was designed to optimize multiplication during four passages.

Dedifferentiated chondrocytes have lost the ability to produce cartilage-specific collagen type II (Benya and Shaffer 1982). To transplant in vitro cultured chondrocytes successfully into an articular cartilage defect, reexpression of collagen type II is essential. Culturing in agarose (Benya and Shaffer 1982, Aulthouse et al. 1989, Shortkroff et al. 1996) and in alginate (Guo et al. 1989, Bonaventure et al. 1994) has been reported to induce redifferentiation of dedifferentiated chondrocytes. We therefore studied the po-

tential of bovine chondrocytes to produce hyaline cartilage matrix after monolayer culture and a subsequent culturing period in alginate.

Methods

Chondrocyte isolation

Full-thickness cartilage slices were harvested, within 8 hours after death, under sterile conditions, from the metacarpophalangeal joints of 12–18-month-old bovine steers, obtained from a local slaughterhouse. The cartilage was washed with sterile physiological saline and incubated with protease XIV (2 mg/mL; Sigma, St. Louis, MO) for 2 hours, followed by an overnight incubation with collagenase B (1.5 mg/mL; Boehringer, Mannheim, Germany) in medium (DMEM/Ham's F12; Gibco, Grand Island, NY) with 10% Fetal Calf Serum (FCS). Both enzymatic digestions were done at 37 °C. After incubation, the undigested cartilage fragments were removed, using a 100 µm filter. The isolated chondrocytes were washed with physiological saline and counted with a hemacytometer. Cell viability was tested with the trypan blue exclusion test. Cells were seeded in 175 cm^2, 75 cm^2 or 25 cm^2 culture flasks or cultured in alginate.

Culture in monolayer

The isolated chondrocytes were seeded in monolayer at a density of 2×10^4 cells/cm^2. The culture medium (DMEM/Ham's F12), supplemented with 10% FCS, Fungizone (0.5 µg/mL) and gentamicin (50 µg/mL), was changed twice weekly.

In an initial experiment, the multiplication of bovine chondrocytes, in response to 1.0 ng/mL TGFß-2 (Sandoz, Basel, Switzerland), 1.0 ng/mL or 10 ng/mL bFGF (Serva, Heidelberg, Germany) and 25 µg/mL L-ascorbic acid (Sigma), was tested in primary culture (P0), in the third passage (P3) and in the tenth passage (P10). 3 culture flasks of 25 cm^2 were used to test each growth factor and 3 flasks were used as controls. When one of the monolayer cultures reached the subconfluent phase, the flasks in all experimental groups were trypsinized (Trypsin-EDTA; Gibco). After washing, the cells were resuspended and two samples of each flask were counted twice with a hemacytometer. Since the interest of this study was cell proliferation over a prolonged period (one passage) incorporation of radiolabeled thymidine was not used. The cells were reseeded in monolayer or suspended in alginate (1.2%; Keltone LV, Kelco, Chicago, IL). Because cell multiplication was stimulated by TGFß-2 in primary culture and by L-ascorbic acid in the third passage, a dose-response experiment was performed.

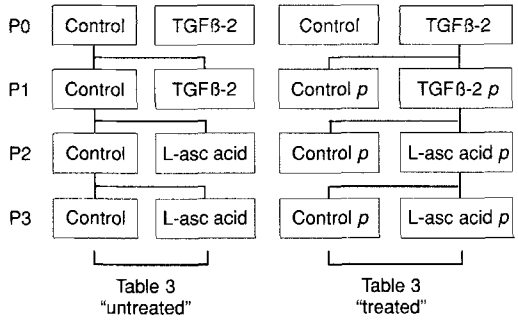


Figure 1. Experimental design of in vitro multiplication of chondrocytes, first treated with TGFß-2, followed by L-ascorbic acid. P0 primary culture, P1 first passage, P2 second passage, P3 third passage. TGFß-2 1.0 ng/mL, L-ascorbic acid 25 µg/L. p = previously incubated.

The culture medium was supplemented with 0.1 ng/mL, 1.0 ng/mL and 10 ng/mL TGFß-2 in primary culture and supplemented with 2.5 µg/mL, 25 µg/mL and 100 µg/mL L-ascorbic acid in the third passage.

Using the results of the above-described experiments, an optimal scheme to administer growth stimulating factors in chondrocyte monolayer cultures was designed. We investigated the response of chondrocytes to 1.0 ng/mL TGFß-2 in primary culture and the first passage and 25 µg/mL L-ascorbic acid in the second and the third passages. Chondrocytes not treated with growth factors in former passages (native chondrocytes) served as controls (Figure 1).

Culture in alginate beads

Cells were encapsulated in alginate beads at a density of $2–4 \times 10^6$ cells/mL. The preparation of chondrocytes in alginate beads was done as described by Guo et al. (1989), with slight modifications, as described by Häuselmann et al. (1992). The cells were suspended in sterile saline containing 1.2% low viscosity alginate gel, then slowly passed through a 21-gauge needle in a dropwise fashion into a 102 mM $CaCl_2$ solution. After instantaneous gelation, the beads were allowed to polymerize further for a period of 10 minutes in the $CaCl_2$ solution. They were thereafter washed with physiological saline and finally plated in a 24-well plate with 7–10 beads/well in 1 mL DMEM/Ham's F12 medium, supplemented with 10% FCS, gentamicin (50 µg/mL), Fungizone (0.5 µg/mL) and 25 µg/mL L-ascorbic acid, freshly added and cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed 3 times a week. After 7, 14, 21 and 28 days, alginate beads were removed from culture and dissolved in 55 mM Na-citrate, supplemented with 5% FCS. After 10 minutes, the cells, with cell-associated matrix (Häuselmann et al. 1994), were cyto-centrifuged onto glass slides at

1000 rpm during 7 minutes. After air-drying at room temperature for 1 hour, the specimens were frozen at –80 °C, until immunohistochemical staining took place.

Immunohistochemical staining

For detection of type I and type II collagen, all slides were initially fixed in acetone for 10 minutes at room temperature and incubated with 1% hyaluronidase (Sigma) for 30 minutes at 37 °C. Nonspecific binding was blocked with 10% normal goat serum. Subsequently, the cells were incubated for 2 hours with monoclonal antibodies to pro-collagen type I (M38 1:100; DSHB, Iowa City, IA) or collagen type II (II-II6B3 1:100; DSHB) at room temperature. The slides were incubated for 30 minutes with a 1:100 dilution of goat Fab-fragment against mouse conjugated with alkaline phosphatase (GAMAP; Immunotech, Marseille, France) in PBS-BSA (1%) supplemented with 10% FCS, followed by incubation for 30 minutes with a 1:100 dilution of mouse monoclonal alkaline phosphatase anti-alkaline phosphatase (APAAP; Dakopatts, Copenhagen, Denmark). The new fuchsin (Chroma, Kongen, Germany) procedure was used for color development. The slides were counterstained with hematoxylin and mounted in gelatin-glycerin. Negative control stainings were done simultaneously by omitting the first antibody. The slides were examined blindly and independently by two observers. The staining for both collagen type I and collagen type II was classified as: (1) less than 10% of the total number of cells staining positive, (2) 10–40% of the total number of cells staining positive, (3) 40–60% positive staining, (4) 60–90% positive staining and (5) more than 90% positive staining.

Data analysis

The experiment to test the response of TGFß-2, L-ascorbic acid and bFGF was performed 3 times. Each experiment consisted of 3 culture flasks per group. The dose-response experiment was performed once with 3 flasks per group, to check data from the literature. The experiment in which the addition of TGFß-2 in the first 2 passages was followed by L-ascorbic acid in the next passages was performed 3 times, with 3 flasks per group. Differences between individual samples were analyzed. One way ANOVA was used to compare multiple groups, a p-value < 0.01 was considered statistically significant. If statistical significant differences were found, the rank sum test was used to compare two experimental conditions, a p-value < 0.05 was considered statistically significant.

Table 1. Effect of TGFß-2, bFGF and L-ascorbic acid on cell proliferation in monolayer (% of control)

Passage	TGFß-2 1.0 ng/mL	bFGF 1.0 ng/mL	L-ascorbic acid 25 µg/mL
Primary	139 (20)	95 (16)	85 (4)
P-value	0.008		0.01
Third	110 (7)	107 (14)	147 (9)
P-value	0.04		0.0004
Tenth	91 (17)	89 (9)	124 (20)

TGFß-2 (1.0 ng/mL), bFGF (1.0 ng/mL) or L-ascorbic acid (25 µg/mL) was added in primary culture (P0), in the third passage (P3) or in the tenth passage (P10) until sub confluency was reached. Data represent mean (SEM) of 3 experiments.

Table 2. Dose-response of TGFß-2 on cell multiplication in primary culture and L-ascorbic acid in the third passage (percentages)

(ng/mL)	Control	0.1	1.0	10
TGFß-2 (P0)	100 (11)	139 (7)	134 (27)	77 (36)
(µg/mL)	Control	2.5	25	100
L-asc acid (P3)	100 (5)	114 (44)	143 (20)	105 (18)

P0 primary culture, P3 third passage.
Cell number of control flasks is set at 100%. Data represent mean (SD) of 3 culture flasks.

Results

After isolation, more than 95% of the cells were viable. In the initial experiment, the time for cells to reach the subconfluent phase was 9 ± 4 days for the primary culture, 5 ± 1 days for the third passage and 7 ± 0 days for the tenth passage. Each passage corresponded to a cell multiplication of 2.4 ± 1.0 times. One experiment was kept in culture until the twenty-second passage. Those cells kept on multiplying with an approximate subconfluent time of one week.

TGFß-2 (1.0 ng/mL) significantly stimulated the proliferation of primary bovine chondrocytes ($p = 0.008$). This stimulation decreased in the third passage ($p = 0.04$) and had disappeared by the tenth passage (Table 1). bFGF (1.0 ng/mL and 10 ng/mL) had no significant effect on the proliferation rate of chondrocytes in any of these passages (Table 1). L-ascorbic acid (25 (µg/mL) had an inhibitory effect ($p = 0.01$) on the proliferation of primary chondrocytes, but became a stimulator in the third passage ($p = 0.0004$) (Table 1).

The dose-response experiment (Table 2) revealed stimulation of multiplication with 0.1 and 1.0 ng/mL

Table 3. Effect on cell proliferation in monolayer culture of the addition of TGFß-2 and L-ascorbic acid on native cells (untreated) or in a sequential addition scheme (treated)

Passage	Untreated	Treated
Primary	130 (9) p = 0.01	130 (9) p = 0.01
First	98 (15)	131 (25)
Second	112 (9)	161 (12) p = 0.0007
Third	132 (9) p = 0.001	128 (13) p = 0.009

For experimental design, see Figure 1. Untreated: Native cells were treated with TGFß-2 (1.0 ng/mL) in primary culture, or in the first passage or with L-ascorbic acid (25 µg/mL) in the second or the third passage. Controls are set at 100%. Treated: TGFß-2 (1.0 ng/mL) was added in primary culture, continued in the first passage and followed by the addition of L-ascorbic acid (25 µg/mL) in the next 2 passages. Controls are set at 100%. Data represent mean (SEM) of 3 experiments.

Table 4. Cumulative multiplication factors of chondrocytes after treatment with TGFß-2 and L-ascorbic acid, compared to native, untreated chondrocytes

Passage	Untreated	Treated [a]
Onset	1.0	1.0
Primary	1.9 (0)	2.5 (0.2)
First	3.1 (0.4)	5.8 (0.8)
Second	8.5 (1.3)	29.5 (3.2)
Third	16 (2.3)	106 (20)

[a] Chondrocytes were treated with TGFß-2 (1.0 ng/mL) in primary culture and the first passage, followed by addition of L-ascorbic acid (25 µg/mL) in the next two passages. Data represent mean (SEM) of 3 experiments.

TGFß-2 and no effect of 10 ng/mL TGFß-2. Addition of 1.0 ng/mL TGFß-2 was chosen because it is widely used in the literature. This experiment also confirmed that the optimal dose of L-ascorbic acid is 25 µg/mL in the third passage.

The above-mentioned results led to the design of an optimal addition scheme, which was tested in the next experiments. As in the previous experiments, TGFß-2 stimulated cell proliferation in primary culture ($p = 0.01$), and L-ascorbic acid stimulated proliferation in the third passage of native, not previously incubated cells ($p = 0.001$) (Table 3, 'untreated'). In the scheme of sequential addition (Table 3, 'treated'), TGFß-2 stimulated in primary culture ($p = 0.01$). In the first passage, two experiments showed stimulation, one experiment did not ($p = 0.2$). A significant stimulating effect of L-ascorbic acid was seen in the second passage ($p = 0.0007$) and in the third passage ($p = 0.009$), when the chondrocytes had previously been incubated with TGFß-2.

The total number of cells in the native control group was 16 times as many as the number of cells to begin with. In the group treated with the above-mentioned optimal addition scheme, the cell number was 106 times higher than the original number of cells (Table 4). This means that 7 times more cells were acquired after treatment with TGFß-2, followed by L-ascorbic acid.

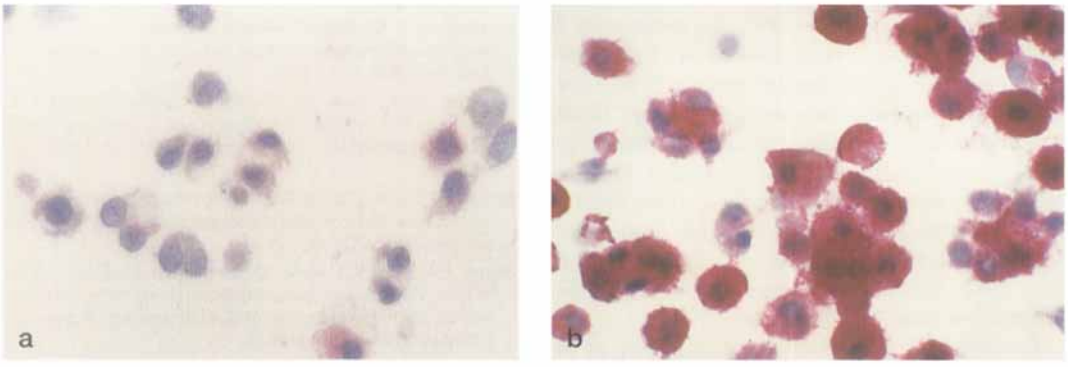
Immunohistochemistry showed that freshly isolated bovine chondrocytes (P0), when cultured in alginate, developed a pericellular matrix, consisting mainly of collagen type II, whereas collagen type I was found in the occasionally formed cell clusters. Chondrocytes suspended in alginate after one passage in monolayer showed collagen type I to be as frequent as collagen type II. The observers found that 50% of the cells were positively stained with the antibody to procollagen type I (M38) and found slightly less than 50% of the cells positively stained with the specific antibodies to collagen type II (II-II6B3). After 3 passages, more than 50% of the cells scored positive for M38 and none of the cells positive for II-II6B3 (Figure 2). The chondrocytes suspended in alginate after 10 or 22 passages in monolayer showed only collagen type I staining. The PBS control sections were all negative. No difference was found between 7, 14, 21 and 28 days of culture in alginate. Addition of growth factors during monolayer did not influence collagen expression in alginate cultures.

Discussion

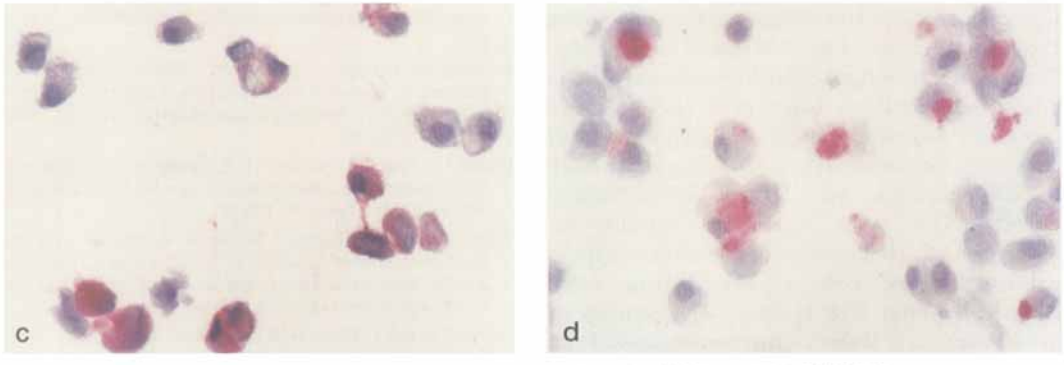
Repair of cartilage defects with autologous chondrocytes requires multiplication of chondrocytes. In this in vitro study, TGFß-2, bFGF and L-ascorbic acid were tested for their ability to stimulate chondrocyte multiplication in monolayer culture. One of the findings in our study is that the response of chondrocytes to TGFß-2 and L-ascorbic changes during cell multiplication. TGFß-2 stimulated the growth of freshly isolated chondrocytes, whereas L-ascorbic acid stimulated chondrocytes in the third passage and inhibited freshly isolated ones. Although we used bovine chondrocytes, we think that these results have important implications in the choice of growth factors to stimulate proliferation of human chondrocytes.

In agreement with our results, the stimulation of chondrocyte proliferation by TGFß has been reported to decrease with the number of in vitro subcultures (Guerne et al. 1994) and may eventually become inhibitory (Blanco et al. 1995). In contrast to our results, bFGF has been reported to stimulate the proliferation of chondrocytes. This conflict may be caused by the different origins of chondrocytes and different

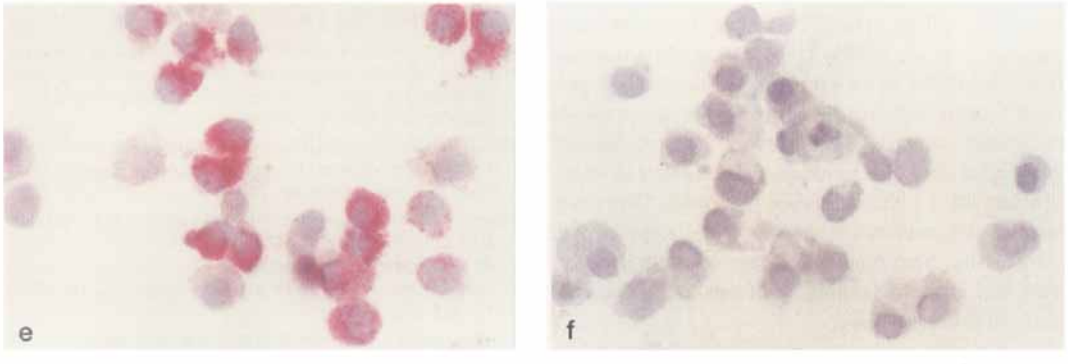
Figure 2. Immunohistochemical staining for collagen type I (M38) and collagen type II (II-II6B3), on chondrocytes cultured in alginate. Illustrating the gradual shift from collagen type II to type I, during the process of cell multiplication. Red staining indicates a positive signal. Magnification ×220.



Freshly isolated chondrocytes, cultured in alginate, stained with M38 (a) and with II-II 6B3 (b).



Cells cultured in alginate, after multiplication in primary monolayer, stained with M38 (c) and with II-II6B3 (d).



Cells cultured in alginate after multiplication in monolayer until the third passage, stained with M38 (e) and with II-II6B3 (f).

culture circumstances (Adolphe et al. 1984, Froger-Gaillard et al. 1989). Our study showed an inhibition of the proliferation of primary chondrocytes by L-ascorbic acid and a stimulation in the third passage which have not been described before.

To obtain as many cells as possible in 4 passages, we chose an optimal addition scheme of growth factors. TGFß-2 was added to the culture medium in the first 2 passages and L-ascorbic acid was added in the subsequent 2 passages. This led to a more than 100-fold increase in the number of cells after 4 passages over 4 weeks. This is 7 times as many cells as obtained in the native, control group. TGFß-2 indeed stimulated proliferation in the first 2 passages. The cells which were treated with TGFß-2 in primary culture and in the first passage were stimulated by L-ascorbic acid in the second passage and in the third passage.

During monolayer culture, chondrocytes dedifferentiate and the number of collagen type II-producing cells decreases (von der Mark et al. 1977, Guerne et al. 1994). This was confirmed by our experiment. If multiplied cells are to be used to repair cartilage defects, they must be stimulated to produce hyaline cartilage specific matrix components. Addition of TGFß-2 and L-ascorbic acid during monolayer culture had no effect on chondrocyte dedifferentiation. This has been reported for TGFß (Galéra et al. 1992, Pujol et al. 1994). Although L-ascorbic acid has been shown to increase dedifferentiation of chondrocytes (Daniel et al. 1984, Aulthouse 1994), we did not observe this effect in our study.

In contrast to the results of Bonaventure et al. (1994), using fetal human chondrocytes, and Benya and Shaffer (1982), using immature rabbit chondrocytes, our chondrocytes did not redifferentiate when cultured in alginate for up to 28 days. Culture in alginate may not be optimal for redifferentiation of adult articular chondrocytes. Yaeger et al. (1997) demonstrated that reexpression of the cartilage phenotype of cells in alginate varied widely, depending on the batch of FCS used. Serum free culturing, with addition of growth factors, was shown to enhance redifferentiation. The potential of our chondrocytes to redifferentiate and produce hyaline cartilage, when cultured in serum free medium, with addition of growth factors, is under current investigation. Even when multiplied chondrocytes cannot be stimulated to redifferentiate in vitro, the cells might still be used to repair cartilage defects. Takigawa et al. (1987) and Shortkroff et al. (1996) showed that chondrocytes, dedifferentiated by in vitro multiplication, can produce hyaline cartilage after in vivo implantation. In conclusion, our study demonstrates that application of growth factors helps to increase the number of chondrocytes after multiplication in monolayer. The study stresses the importance of adding the right growth factor at the right moment. Taking this into account, we could increase the number of cells more than 100 times in about 4 weeks.

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