Long-term effects of osteogenic protein-1 on biosynthesis and proliferation of human articular chondrocytes

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Abstract

Objective

To study the long-term effects of OP-1 on the biosynthesis and proliferation of human articular chondrocytes.

Methods

Chondrocytes were released from human cartilage from 10 organ donors of different ages and cultured in alginate. They were exposed to OP-1 (0-200 ng/ml) for 3 to 60 days. Proteoglycan (^{35}S-sulfate) and collagen (^{3}H-proline) synthesis were measured by radiolabeling. Proteoglycan content was determined by a dimethylmethylenblue assay, hydroxyproline content by a colorimetric assay, and DNA content by a fluorometric assay.

Results

Long-term (60 days) cultures of human adult articular chondrocytes stimulated by OP-1 (50 ng/ml) revealed a relative decrease of proteoglycan and collagen synthesis. However, proteoglycan (5-fold) and collagen (1.4-fold) content were increased even after 60 days in culture when compared to controls. Maintaining the chondrocyte phenotype (aggrecan synthesis as the main proteoglycan) in long-term culture, OP-1 (50 ng/ml) stimulated proliferation up to 2.4-fold.

Conclusion

Maintaining a stable phenotype and accelerating matrix assembly and proliferation in long-term culture OP-1 might support the tissue engineering of human cartilage.

Key words

Human articular chondrocytes, biosynthesis, proteoglycan, collagen, long-term culture.

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Introduction

Osteogenic protein-1 (OP-1), also referred to as bone morphogenetic protein-7 (BMP-7), is a member of the TGF-ß superfamily. Various studies have elucidated the effect of different BMPs on animal articular chondrocytes *in vitro* (1-3). Chen *et al.* (4) provided evidence that OP-1 stimulates matrix synthesis in bovine articular cartilage explants without causing hypertrophy of the chondrocytes. Luyten *et al.* (5) demonstrated increased levels of expression of type II collagen and aggrecan (as phenotypic markers) in short-term cultures of bovine articular chondrocytes.

Flechtenmacher *et al.* (6) published that OP-1 stimulates proteoglycan synthesis in 3-day cultures and collagen synthesis in 10-day cultures of human articular chondrocytes without affecting their proliferation. Furthermore, OP-1 is able to overcome IL-1ß induced suppression of proteoglycan synthesis by human articular chondrocytes (7). Nishida *et al.* (8) demonstrated enhanced hyaluron deposition, increased aggrecan synthesis (5-day culture) and increased expression of CD44 at the cell surface by OP-1 stimulated chondrocytes.

Häuselmann *et al.* (9) demonstrated phenotypic stability of human articular chondrocytes in long-term alginate cultures. Lietman *et al.* (10) have shown stimulation of proteoglycan synthesis in explants of porcine articular cartilage after OP-1 treatment up to 10 days under serum free conditions.

Sailor *et al.* (11) demonstrated relatively high levels of mRNA expression for aggrecan and collagen type II by BMP-2 treated bovine chondrocytes in 28-day monolayer cultures compared to controls. However, cell morphology laid open two subpopulations in the BMP-2 group: one revealed the typical rounded morphology, while the other appeared elongated and fibroblast-like. After 28 days controls and BMP-2 treated cultures showed equal cell numbers.

Since long-term observations are essential and phenotypic stability of the chondrocytes is very important for tissue engineering and applications *in vivo*, the present study elucidates the effects of OP-1 on long-term (60 days) cultures of human articular chondrocytes in alginate. Total proteoglycan and collagen synthesis and content, and chondrocyte proliferation were determined.

Materials and methods

Reagents

All chemicals were of regular grade and purchased from Sigma (St. Louis, MO) unless otherwise noted. Pronase was purchased from Calbiochem, La Jolla, CA, collagenase P (Clostridium histolyticum) from Boehringer Mannheim, Indianapolis, IN. Low viscosity alginate (Keltone LV) was a gift from Kelco, Chicago, IL. Ham's F-12 medium (F12) and Dulbecco's modified Eagle's medium were from Gibco, Grand Islands, NY. FBS was obtained from HyClone, Logan, UT, and Gentamicin from Life Technologies, Grand Island, NY. Recombinant human OP-1 was kindly provided by Dr. Sampath (Creative BioMolecules, Massachussets). ^{35}S-sulfate (25-40 Ci/mmol, 0.9-1.5 TBq/mmol) and L-(2,3,4,5-3H) proline (85-130 Ci/mmol, 3.15-4.81 TBq/mmol) were purchased from Amersham Corporation, Arlington Heights, IL. Hydrofluor was purchased from National Diagnostics, Manville, NJ. Alcian blue was obtained from Bio-Rad, Richmond, CA. 96-well filter plates, a vacuum manifold and punch tip assemblies were purchased from Millipore, Bedford, MA. Sephadex G-50, DEAE-Sephacel and Sepharose CL-2B columns were obtained from Pharmacia LKB Inc., Piscataway, NY. Electrophoresis reagents, polyacrylamide, bis-acrylamide, sodium dodecyl sulfate, N,N,N',N'-tetramethylenediamine, and glycine, were purchased from Bio-Rad Laboratories, Melville, NY. Bisbenzimidazole fluorescent dye (Hoechst dye # 33258) was obtained from Polysciences, Warrington, PA.

Tissue acquisition

Non-calcified articular cartilage from knee joints and one ankle joint was obtained from 11 organ donors without known joint disease within 24 hours of death through the Regional Organ

Bank of Illinois according to their protocol and with institutional approval: two third-trimester fetuses, a newborn, an 11-week, a 3-year, a 5-year, a 20-year, a 28-year (ankle and knee joints), a 29-year, a 52-year and a 68-year old.

Preparation of alginate beads
Cartilage slices were either used for organ culture (28-year-old donor) or were digested with 0.4% pronase and 0.02% collagenase (12 ml/g cartilage) under gentle conditions at 37°C overnight (12,13). The released chondrocytes were rinsed, filtered and counted. Isolated chondrocytes were then suspended in 1.2% alginate. By dropping the chondrocyte-suspension into isoosmotic calciumchloride-solution (102 mM) alginate-beads containing approximately 40,000 chondrocytes were formed (13).

Culture
Isolated chondrocytes embedded in alginate and organ cultures were maintained in culture medium for 5 days to allow reconstitution of their matrix. Chondrocytes were fed daily with basic medium, containing Dulbecco's modified Eagle's medium (45%), Ham's medium F12 (45%), 10% FBS, 25 µg/ml ascorbate, 10 µg/ml gentamicin. Experiments were carried out in triplicates.

Treatment with OP-1
Chondrocytes were treated daily with basic medium containing 10% FBS (if not noticed differently) in the presence or absence of OP-1. Concentrations of OP-1 (molecular weight about 34 kD) ranging from 50 to 200 ng/ml (1.47 - 5.88 nM) were used since dose response studies showed a high sensitivity of human articular chondrocytes to these concentrations. However, the concentration of OP-1 in human serum varies between 0.5 and 1 ng/ml, and the physiologic concentration in human articular cartilage is unknown (6).
OP-1 was maintained in a stock solution with 1 mg OP-1/ml [vehicle: 50% ethanol, 0.1% trifluoroacetic acid (TFA)] at -20°C (14). In 50 ng OP-1/ml (200 ng/ml) containing media the final concentrations were: 2.5×10^{-3} % (0.01%) for ethanol, and 5×10^{-6} % (20×10^{-6} %) for trifluoroacetic acid.

Proteoglycan analysis
In the last 4 hours of cytokine treatment cultures were labeled with ^{35}S-sulfate (20 µCi/ml) to evaluate proteoglycan (PG) synthesis. The cultures then were processed according to the published protocol (13). The amount of all newly synthesized proteoglycans was measured by a rapid filtration assay using the binding capacity of proteoglycans to alcian blue (15).
To ascertain that OP-1 is stimulating mainly aggrecan synthesis samples of a long-term experiment (OP-1 treatment for 3, 10 and 60 days) using the knee cartilage from a 52-year old donor were lyophilized after the DEAE-Sephacel column purificaton, equal amounts of radioactivity (exception: sample of day 60 of control group) dissolved in sample buffer and then loaded on a gradient (4-20%) SDS-PAGE (13, 16).

Collagen extraction and quantitative analysis of collagens via HPLC
At various time points (18, 30, and 60 days) collagens were labeled for 48 hours with ^{3}H-proline (20 µCi/ml medium). Afterwards, proteinase inhibitors were added, and the beads dissolved. Collagens were extracted overnight at 4°C. The samples were then dialyzed at 4°C overnight.
Lyophilized ^{3}H-proline-labeled samples were hydrolyzed in 6 N HCl at 110°C for 18 h. ^{3}H-hydroxy-proline and ^{3}H-proline were separated by HPLC on a Vydac Sulfonyl Cation-Exchange column using the same buffer. Radiolabeled fractions containing ^{3}H-hydroxyproline (7.0 min.) and ^{3}H-proline (12.0 min.) were counted in a liquid scintillation counter (17).

Proteoglycan and collagen content
Proteoglycan content was determined by binding to dimethylmethylene blue (DMB) dye in the presence of 0.24 M GuHCl since this prevents precipitation and interference of hyaluron acid and / or DNA as described by Chandrasekhar et al. (18). Absorbance was measured at 530 and 595 nm using the Kineticalc program on the Bio-Tek Microplate reader.
Hydroxyproline content was measured by a colorimetric assay as described by Schwartz *et al*. (19).

DNA content
DNA content was measured in aliquots of the sodium citrate dissolved beads after papain digestion by a modified fluorometric assay (emission measured at 400-550 nm for an excitation wavelength of 365 nm) using bisbenzimidazole dye (Hoechst 33258) (20).

Statistical analysis
Data are expressed as mean ± standard error. The statistical significance of differences between group means was determined by Student's t-test. $P < 0.05$ was considered statistically significant.

Results
PG synthesis
On day 10 human articular chondrocytes revealed their strongest response to OP-1 with a 6.5 (50 ng/ml) up to 8.6 (200 ng/ml OP-1) increase of PG synthesis (Fig. 1a). The lower concentration of OP-1 did not achieve a stimulation of PG synthesis after 2 months in culture, whereas OP-1 in a concentration of 200 ng/ml could still slightly increase PG synthesis compared to control cultures (Fig. 1a). Fluorograms confirmed that aggrecan was the major proteoglycan synthesized in all experimental groups (Fig. 1b). Due to its size, aggrecan (total size $1.5\text{-}2.5 \times 10^{6}$ D) barely penetrated the gel. However, the control group already revealed some synthesis of small proteoglycans (biglycan and decorin) after 3 and 10 days. After 60 days small proteoglycans could be detected in the OP-1 treated groups (Fig. 1b). Missing bands of small proteoglycans for the control group on day 60 were most likely due to the lower amount of loaded counts (lane 1*, Fig. 1b).

Collagen synthesis
Collagen synthesis (^{3}H-Hydroxyproline) was long-term upregulated (up to 5.9-fold) by OP-1 in chondrocytes derived from a fetal (Fig. 2a) and an 11-week-old donor (Fig. 2b). However, 60-day cultures of adult chondrocytes

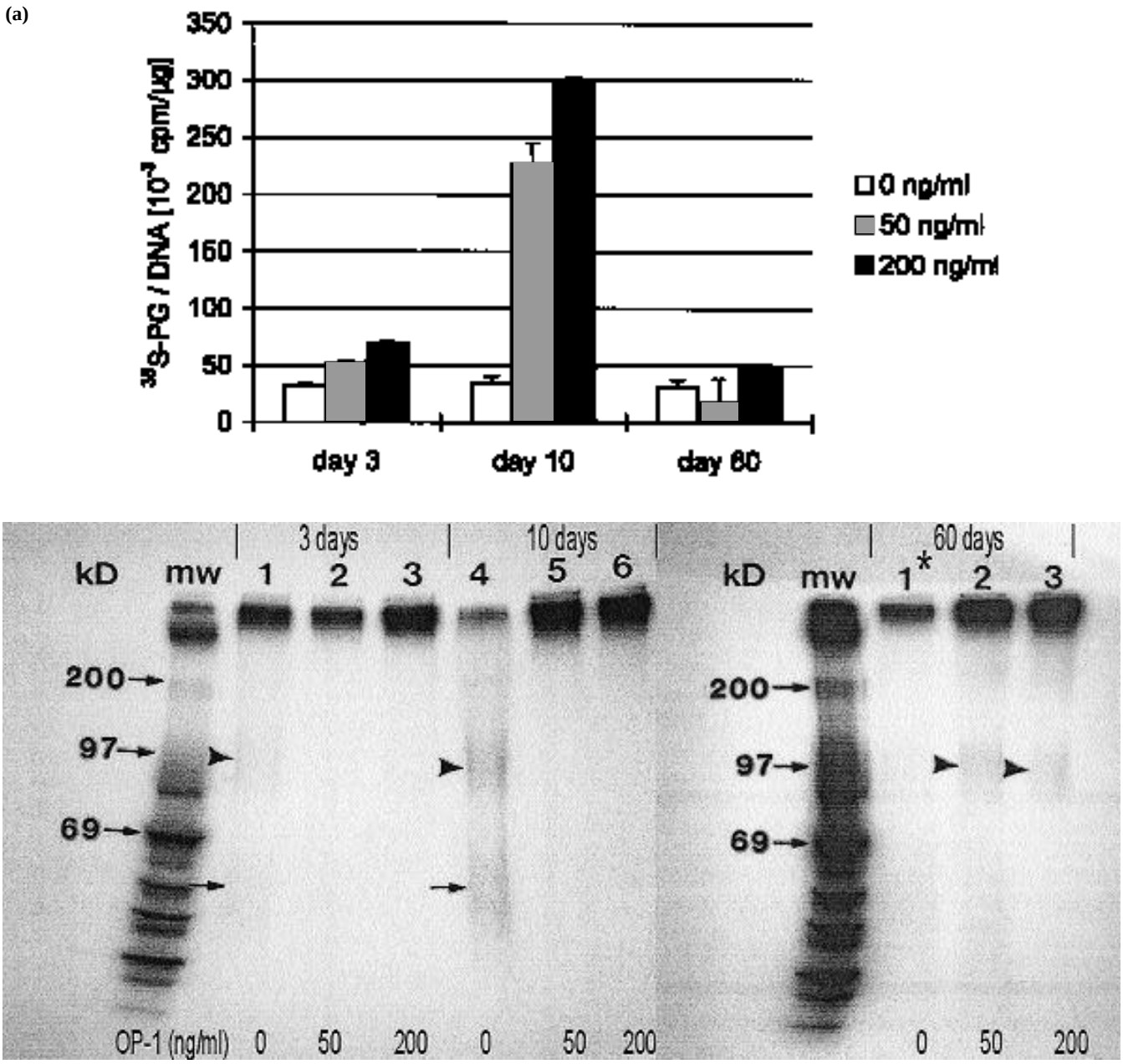


Fig. 1. (a) Effect of OP-1 (50 or 200 ng/ml) on ^{35}S-PG-synthesis by articular chondrocytes from a 52-year-old donor after 3 to 60 days of treatment. Values are given as mean ± standard deviation (stdev); $p < 0.05$ when compared to controls of each time point. **(b)** SDS-PAGE (4-20%) of ^{35}S-labeled proteoglycans synthesized by chondrocytes obtained from the same donor as in (a). Equal counts were loaded with the exception of lane 1*, where 32% less cpm could be loaded to the gel compared to all other lanes. Aggrecan (around 2 x 10^6 D) barely penetrated the gel (13). Arrows mark bands corresponding to about 97 kD (e.g. biglycan) and to about 36 kD (e.g. decorin) (22).

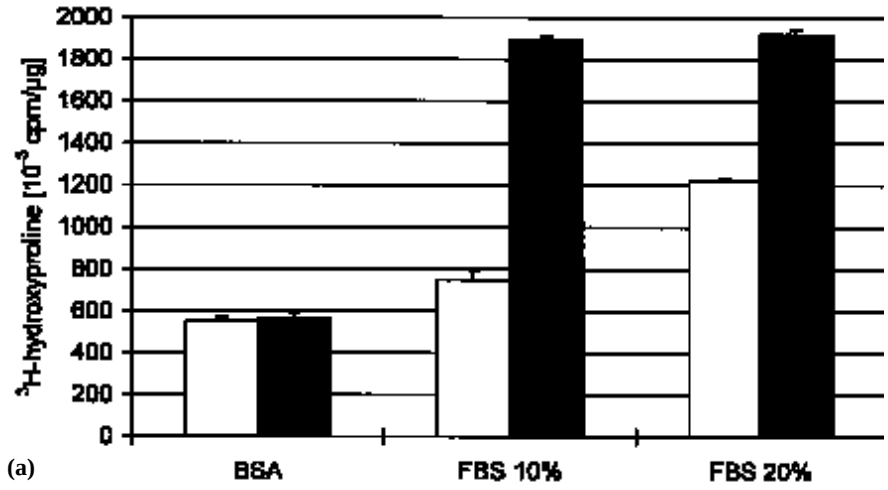
(28- and 29-year-old donors) showed a decrease (down to 0.3-fold) of collagen synthesis after treatment with OP-1 in comparison to control cultures (Fig. 2b, c). In 30-day cultures of fetal chondrocytes OP-1 stimulated collagen synthesis in the presence of 10% or 20% FBS compared to OP-1- and FBS-free controls (Fig. 2a).

Effect on proteoglycan and collagen content

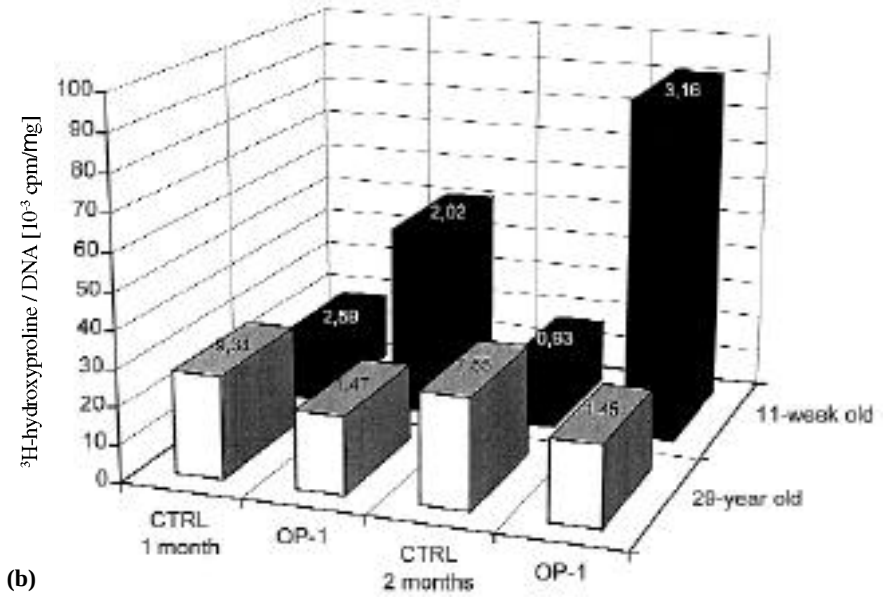
Since PG and collagen synthesis were reduced in long-term cultures of adult chondrocytes compared to mid-term cultures (10 to 18 days), PG and collagen content were determined for adult chondrocytes (20- and 29-year-old donors) after 7, 30 and 60 days in culture in the presence or absence of OP-1 (10, 50 and 200 ng/ml). For all time points and doses the content of both matrix molecules was higher for OP-1 treated groups. After 60 days PG content was increased about 5-fold and the collagen content by about 1.4-fold compared to controls (Table I).

Effect on chondrocyte proliferation

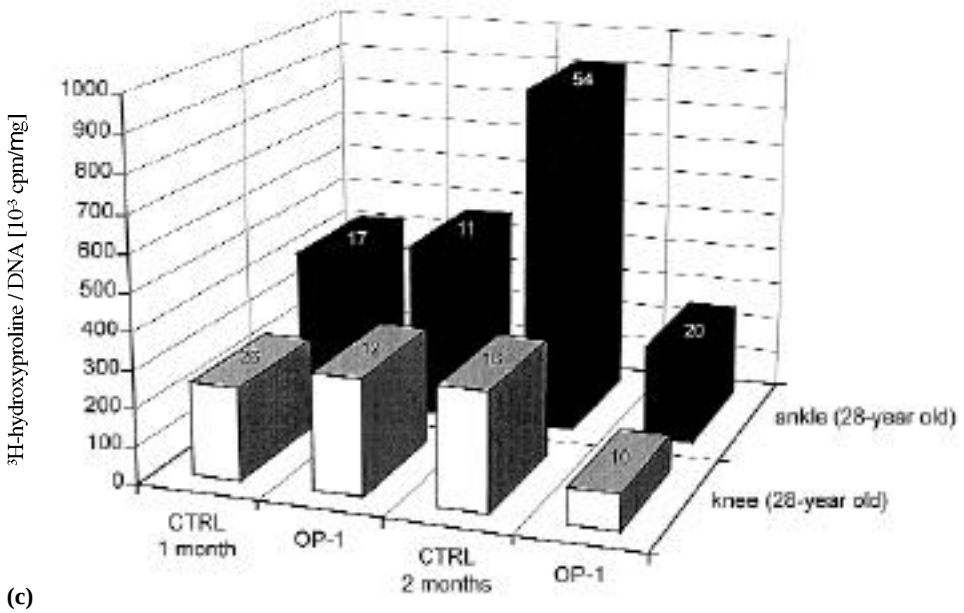
In cultures of articular chondrocytes



(a)



(b)



(c)

derived from joints of different aged donors, OP-1 did not substantially change the proliferation rate of the human chondrocytes during the first days of treatment compared to controls. However, after 18 and 60 days 50 ng OP-1/ml significantly stimulated cell proliferation up to about 2.4-fold. 200 ng OP-1/ml seems to be more effective since it increased chondrocyte proliferation in long-term cultures up to 3.6-fold (Table II).

Discussion

The results of this study reveal that OP-1 (200 ng/ml) can effectively stimulate proteoglycan synthesis not only in short-term (3 to 10 days) but also in long-term cultures (60 days). The phenotypic stability of the chondrocytes is supported by their microscopically normal shape and their main synthesis of aggrecan. The expression of small amounts of small proteoglycans can be regarded as physiologic (13). Häuselmann *et al.* (13) have shown similar results for alginate cultures maintained in basic medium containing 10% FBS. Therefore, our results underline the role of alginate as an artificial scaffold for the restoration of chondrocyte matrix after chondrocyte isolation from natural grown cartilage.

Concerning collagen synthesis, *adult* chondrocytes in long-term culture seem to be less sensitive to OP-1 than

Fig. 2. **(a)** Collagen synthesis (^{3}H-hydroxyproline) by OP-1 (100 ng/ml) treated fetal chondrocytes in the presence of different concentrations of FBS (0%, 10%, or 20%) after 30 days. $p < 0.05$ when compared to controls
(b) Collagen synthesis (^{3}H-hydroxyproline) by OP-1 (50 ng/ml) treated chondrocytes (11-week old and a 29-year old donor) after 30 and 60 days. $p < 0.05$ when compared to controls (CTRLs) (means as bars, standard deviations as numbers)
(c) Collagen synthesis (^{3}H-hydroxyproline) by OP-1 (100 ng/ml) treated adult chondrocytes (ankle and knee cartilage slices from a 28-year old donor) after 30 and 60 days. $p < 0.05$ when compared to controls (means as bars, standard deviations as numbers).

Table I. Proteoglycan and collagen content in alginate cultures of human articular chondrocytes derived from knee chondrocytes of a 20-year-old and a 29-year-old donor after 7, 30 and 60 days in the presence of OP-1 (0, 10,50, 200 ng/ml) as % of controls ($p < 0.05$ when compared to controls).

Donor [age]	OP-1 [ng/ml]	Time [days]	Proteoglycan [%]	Collagen [%]
20-year	10	7	112	168
	50		177	256
	200		198	234
29-year	50	30	449	141
		60	488	135

Table II. DNA content in OP-1 (50 ng/ml) treated alginate cultures of articular chondrocytes in percent of appropriate controls (for each donor and time point). $P < 0.05$ when compared to controls, $*p > 0.05$.

Donor [age]	OP-1 [ng/ml]	Day 10	Day 18	Day 30	Day 60
Newborn	50		181		
11-weeks	50	152		203	213
3-years	50		138		
5-years	50		123		
29-years	50	100*		130	130
	200			190	
52-years	50	120			238
	200	151			358
68-years	200			176	

young chondrocytes. This observation is similar to that by Luyten *et al*. (5), who showed that BMP-4, TGF-ß and activin A are more effective in stimulating proteoglycan synthesis by *young* bovine chondrocytes than by *adult* articular chondrocytes. The same study revealed that the numbers and affinity of the BMP-4 binding sites on the chondrocytes were not related to the age of the bovine donors.

Since proteoglycan and collagen *con - tent* are increased in long-term cultures of OP-1 (50 ng/ml) stimulated chondrocytes from adult donors, the decrease in proteoglycan and collagen *synthesis* after 60 days in culture might have been caused by a feed-back mechanism due to a relatively dense neomatrix around the alginate embedded chondrocytes. However, higher concentrations of OP-1 (200 ng/ml) stimulate proteoglycan synthesis and proliferation of adult chondrocytes after 60 days compared to controls, indicating that this concentration is more powerful in long-term cultures of adult chondrocytes.

As shown by Chen *et al*. (4) for monolayer or micromass cultures of bovine articular chondrocytes and by Flechtenmacher *et al*. (6) for alginate cultures of human articular chondrocytes, OP-1 does not significantly increase the proliferation rate during the first 3 to 4 days of culture. However, after more than a week we have found a marked increase in proliferation for both, newborn and adult human articular chondrocytes stimulated by OP-1.

In conclusion, OP-1 appears to be a very effective stimulator of PG and collagen synthesis by human articular chondrocytes in short- and long-term culture. In long-term culture, 200 ng OP-1/ml seems to be an effective concentration for adult chondrocytes, whereas young chondrocytes seem to be sensitive to 50 - 100 ng OP-1/ml. Since aggrecan is the major PG synthesized under stimulation by OP-1, OP-1 stimulated articular chondrocytes appear to preserve their phenotype. Taking into account that OP-1 is also able to overcome IL-1 suppression of proteoglycan synthesis for concentrations up to 10 pg/ml (7) it can be considered as a possible candidate for the initiation of cartilage repair in joints with circumscribed cartilage lesions or for the assembly of cartilage matrices *in vitro*. Since osteoarthritic cartilage contains less mature OP-1 than normal cartilage (21) OP-1 could also play an important role in the homeostasis of articular cartilage.

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