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Ascorbate modulation of bovine chondrocyte growth, matrix protein gene expression and synthesis in three-dimensional collagen sponges

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Abstract

This report completes a previous study on the growth and metabolism of fetal bovine epiphyseal chondrocytes cultured, within native or cross-linked collagen sponges carried out without the addition of fresh ascorbate. At low initial cell density (2.3×10^6 cells/cm^3) cell proliferation and a low matrix deposition were observed, whereas at high initial cell density (2.3×10^7 cells/cm^3) there was an absence of cell proliferation, but the deposition of a cartilage-like matrix was measured. In both cases, only traces of type I collagen (marker of chondrocyte dedifferentiation) were detected. In this report, we observed, after 1 month in culture with ascorbate, in both type of scaffolds and initial cell densities, an increase in cell proliferation (2-fold) and in expression of genes encoding for collagen types I, II, X and MMP-2 and -13, but no change in the level of matrix deposition (collagen and GAG). With regard to the proteins present, the main differences with or without ascorbate concerned the increase of neosynthesised type I collagen (up to 35% of the total collagen deposited in the sponge) and of the MMP-2 active form. In conclusion, these results show that ascorbate is an important factor to consider when preparing cartilage constructs for its action on chondrocyte phenotype modulation and proliferation.
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Keywords: Ascorbate; Bovine chondrocytes; Collagen biomaterials; Cartilage engineering

1. Introduction

The inability of cartilage to self-repair to any significant degree has been extensively studied and reported by numerous investigators [1–7]. Clinical procedures used to treat the articular cartilage defects include lavage and debridement, drilling, microfracture, and abrasion arthroplasty, all of which cause formation of a fibrocartilage lacking the mechanical properties of hyaline cartilage. Since the pioneering work of Brittberg et al. [8] autologous chondrocyte transplantation (ACT) is now a widely used technique for the treatment of deep cartilage defects of articular cartilage [9–12]. In order to improve retention of cells in the defect and/or to transplant differentiated or in vitro redifferentiated chondrocytes, different scaffolds and matrices have been proposed for in vitro chondrocyte culture before transplantation. Several cell types (chondrocytes derived from auricular, nasal or articular cartilage or progenitor cells) have been cultured under different conditions after seeding in natural (fibrin [13–19], alginate [19–26], agarose [27–28], hyaluronan [29–33] or collagen [34–50]) or synthetic (PGA/PLA, polyethylene or polyethylene oxide or glycol) biomaterials [51–67]. In a previous study [49] we evaluated the ability of collagen-based matrices, in the form of native or cross-linked (DPPA) sponges, to support the growth of fetal bovine epiphyseal chondrocytes, without changes in their initial phenotype, leading to the formation of a cartilage construct with a composition as close as possible to that of natural cartilage. Under these culture conditions, characterised by the lack of addition of fresh ascorbic acid to the culture medium (RPMI/NCTC (v/v) containing 10% fetal calf serum) and the use of static flasks, we observed after 4 weeks (i) a 4-fold increase in cell number and a low

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level of matrix deposition in sponges seeded with 10^6 cells, (ii) an absence of cell proliferation and the neoformation of a cartilage-like matrix in sponges seeded with 10^7 cells. In both cases only traces of type I collagen (marker of chondrocyte dedifferentiation) were detected. These findings demonstrated the suitability of collagen-based scaffolds for cartilage tissue engineering as well as the necessity to improve the culture conditions in order to obtain a better quality cartilage construct (for example higher collagen and glycosaminoglycan contents for sponges seeded with small numbers of cells). In another set of experiments [68–71] using high density culture (10^6 cells/cm^2) of the same cells (fetal bovine chondrocytes) on plastic, we noted [71] that the addition of fresh ascorbic acid (25 μg/ml) to the culture induced increases in both cell proliferation and total protein and collagen synthesis as well as a progressive cell maturation (type I and X collagen synthesis after 30 days). After comparing these results [49,71], we decided to examine the influence of fresh ascorbic acid addition on fetal bovine chondrocyte proliferation and metabolic response after culture for 1 month within native and DPPA-treated type I collagen sponges. Here we describe the chondrocyte response (with initial seedings of 10^6 and 10^7 cells per sponge) by quantitation of DNA content as well as gene expression of collagens I, II and X and matrix metalloproteinases-2 and -13 (MMP-2 and -13), by RT-PCR on RNA isolated after 1 month. We also measured the levels of total collagen and collagens I and II neosynthesised after 7 and 30 days, the level of active and inactive MMP-2 after 7, 15 and 30 days and the content of sulphated glycosaminoglycans deposited in the matrix after 30 days.

2. Materials and methods

2.1. Collagen sponges

Collagen scaffolds were made as previously described [49]. In brief, collagen sponges, manufactured by Coletica (Lyon, France), were made up of collagen extracted from the skin of young calves containing 90–95% (dry weight) native type I collagen and 4–9% type III collagen. Control sponges (denoted by H) and DPPA sponges (control sponges cross-linked using the diphenylphosphorylazide method [49,72]; denoted by D) were used in this study. Individual discs were cut out with a 10 mm diameter punch before being sterilised with 15 kGy β radiation.

2.2. Chondrocyte seeding onto collagen scaffolds

After enzymatic isolation, fetal bovine chondrocytes were cultured as previously described [49]. Briefly, each sponge (10 mm diameter × 6 mm thick disc) was seeded with 10^6 or 10^7 chondrocytes deposited onto the sponge in a volume of 50 μl, then further incubated in 24-well culture plates at 37°C with 5% CO_2 for 2 h. Subsequently, 2 ml of complete culture medium [RPMI/NCTC (v/v) medium (Seromed®, Strasbourg, France/Sigma, Saint Quentin Fallavier, France) containing 10% fetal calf serum (Seromed®), 50 U/ml penicillin, 50 μg/ml streptomycin and 1 ng/ml amphotericin] were added to each well. The sponges were maintained in culture for 48 h to allow the cells to attach to the scaffold.

2.3. Cell-seeded sponge cultivation

Sponges were cultured in 24-well plates for 1 month, changing the medium every 3 days. In some experiments, fresh L-ascorbic acid was added to the medium throughout the culture at various concentrations: 8 mg/l (0.05 mM) for the first 24 h, 10 mg/l (0.06 mM) between 24 and 48 h and 25 mg/l (0.14 mM) thereafter. Throughout the study, unseeded sponges were maintained in culture as controls.

2.4. Cell proliferation

Numbers of chondrocytes per sponge were calculated from the amounts of DNA measured using the Hoechst 33258 dye [73], based on the known DNA content per chondrocyte of 8 pg [71].

2.5. Collagen synthesis and typing

Collagen synthesis was measured on days 7 and 30 by labelling the culture with 20 μCi per sponge of ^{35}S methionine (1000 Ci/mmol, SJ 204 Amersham Biosciences, Orsay, France) for 14 h in fresh RPMI lacking methionine and in the absence of fetal calf serum, as previously described [69]. After labelling, the medium and washed sponges were dialysed separately against water to eliminate unincorporated radiolabel. Samples were frozen, freeze-dried and submitted separately to limited pepsin digestion as previously described [49]. Pepsin treatment with magnetic stirring almost totally solubilised the sponges. The content of dpm in the pepsin-treated samples gave the level of total protein synthesis while pepsin-resistant proteins, assumed to be collagenous, were retained following dialysis. After lyophilisation or acetone precipitation (volume ratio 1:5) samples were dissolved in Laemmli's buffer and counted for their radioactive content.

Pepsin-digested samples were analysed by SDS-PAGE using 6% separating gels under non-reducing conditions, as previously described [49]. Volumes corresponding to the same amount of material were loaded on each lane of the gel. Bands corresponding to types I, II and XI collagens were visualised by fluorography and their relative proportions were quantified densitometrically on the fluorographs with a

Personal Densitometer (Amersham Biosciences) using Image Quant software as previously described [69].

2.6. Glycosaminoglycan content

Amounts of sulphated GAG deposited in the cultured sponges were determined spectrophotometrically at 525 nm using dimethylmethylene blue dye and shark chondroitin sulphate (Sigma) as standard [74].

2.7. Metalloproteinase analysis

Gelatinase activity was measured by gelatin-substrate zymography as described [75] using the culture medium from bovine chondrocytes grown in collagen sponges for 24 or 48 h in RPMI/NCTC medium without fetal calf serum. From each culture medium 5–10 μl samples were mixed with 4X Laemmli's loading buffer and subjected to electrophoresis in 8% polyacrylamide gels containing 0.1% gelatin. After electrophoresis, gels were washed with 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl and 2.5% Triton X100 then incubated at 37°C for 16 h in 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl, 10 mM $CaCl_2$. Gels were stained with 0.5% Coomassie brillant blue R-250, then destained and analysed using a Personal densitometer and Image Quant software (Amersham Biosciences).

2.8. Histology and immunochemistry

Samples were extensively rinsed in phosphate-buffered saline (PBS), fixed for 24 h with 2.5% paraformaldehyde in PBS prior to inclusion in Tissue Tek OCT. For general evaluation, sections (5–7 μm thick) were stained with hematoxylin-eosin. Proteoglycan staining was performed using safranin-O. For immunofluorescence staining, cryostat sections were post-fixed in 2.5% paraformaldehyde in PBS for 1 h, rinsed in PBS, treated with 0.2% hyaluronidase (Sigma Type III, 800 U/mg) in PBS for 1 h at 37°C then incubated for 1 h in 1% bovine serum albumin before treatment with monoclonal antibodies. Antibodies against chicken type-II collagen (Neomarkers, Interchim, Montluçon, France) which also recognise bovine protein and monoclonal antibodies against bovine type-I collagen (Sigma) were used in this study. Samples were then incubated for 1 h at room temperature with the primary antibodies diluted in PBS/1% BSA (1:100 for anti-type-II and anti-type-I collagen), then washed with PBS and incubated with fluorescein-conjugated goat anti-mouse serum. Samples mounted in glycerol/PBS (1/1) were examined using a Zeiss Universal microscope equipped for epifluorescence.

2.9. Total RNA extraction

RNA was extracted from the construct after 1 month in culture using a RNeasy kit (Qiagen, Courtabœuf, France). Additional steps included protein digestion using proteinase K and on-column DNase digestion using RNase-free DNase set (Qiagen). RNA was eluted from the membrane with diethylpyrocarbonate-treated water. RNA purity was confirmed spectrophotometrically (260/280 ratio) and ranged between 1.6 and 2.1.

2.10. Reverse transcription and polymerase chain reaction (RT-PCR)

Reverse transcription of 1 μg of total RNA was carried out in a 40 μl reaction volume with 200 U of SuperScript II RNase H^- (GibcoBRL/Life Technologies, Cergy Pontoise, France) under the following conditions, previously described [76]: 42°C for 50 min and 70°C for 15 min, cDNA was then submitted to RNase H digestion using 4 units of enzyme (GibcoBRL/Life Technologies) for 30 min at 37°C. For PCR, specific primers were designed for the Col1a1, Col1a2, Col2a1, Col10a1, MMP-2, MMP-13 and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes according to their respective sequences (see Table 1). Each 50 μl volume reaction contained: 1 μl RT product, 50 μM of each dNTP, 0.2 μM each primer, 0.5–1.5 mM $MgCl_2$ and 2.5 U Amplitaq DNA polymerase (Applied Biosystems, Les Ulis, France). A denaturation step of 2 min at 94°C was carried out before the amplification step which consisted of 23–40 cycles (23 cycles for GAPDH, Col1a2 and Col2a1, 31 cycles for MMP-2 and 40 cycles for MMP-13, Col10a1 and Col1a1) of 30 s at 94°C, 30–45 s at the annealing temperature, 45 s at 72°C followed by a final extension step of 5 min at 72°C. All PCR products were subcloned into the pCR 2.1-TOPO vector (Invitrogen, Groningen, The Netherlands) and subjected to sequencing for their identification (Genome Express SA, Grenoble, France). Amplified products were analysed by electrophoresis on 2% agarose gels followed by staining with ethidium bromide. The amplification signal obtained for the *GAPDH* gene was used to normalise the amount of cDNA in each sample.

3. Results

3.1. Cell proliferation

Total amounts of DNA were determined in sponges seeded with 10^6 and 10^7 chondrocytes cultured in control (H) and cross-linked (D) scaffolds and in medium either supplemented or not with ascorbic acid. No significant differences were observed between

Table 1
Nucleotide sequences of primers used for RT-PCR

Genes	Primers	Strand	Product size (bp)	Annealing temperature (°C)	Reference
GAPDH	ATCACTGCCACCCAGAAGAC	+	443	57	Bluteau et al. (2001)
	ATGAGGTCCACCACCCTGTT	–			
Col1a1	CACCTACCACTGCAAGAACAG	+	513	45	Personal data
	GAATGCACTTTTGGTTTTTGGTG	–			
Col1a2	GGTTACTACTGGATTGACCC	+	425	55	AB008683
	GCAGCCATCTACAAGAACAG	–			
Col2a1	GATCCGCAACATGGAGACTGGCGA	+	527	75	Personal data
	CAAGAAGCAGACAGGCCCTATGTCCAC	–			
Col10a1	GCAACAGCATTATGACCCA	+	340	51	BTCOL10A1
	CACCAAAGGAAGCCATCG	–			
MMP-2	GGAACAGATCACATACAGG	+	414	51	AF135231
	CACCAAAGGAAGCCATCG	–			
MMP-13	GATGCCATAACCAGTCTCC	+	488	51	AF072685
	GCTGTATTCAAACTGTATGG	–			

Primers are presented in the 5′-3′ orientation. No sequences for *Col1a1* and *Col2a1* are available in the databank; oligonucleotides were designed from sequences determined in our laboratory. GAPDH primers were obtained from the literature.

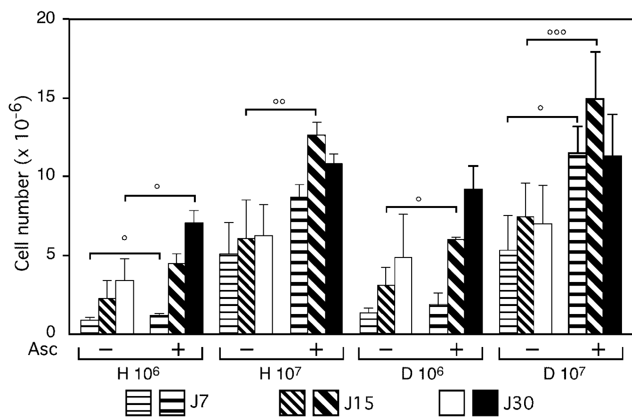

Fig. 1. Effect of ascorbic acid on the proliferation of bovine chondrocytes seeded in collagen sponges. Cells were seeded in either control sponges (H) or in DPPA sponges (D) at one million (10^6) and 10 million (10^7) cells per sponge. Cell numbers were quantified by measuring the DNA content of the sponges after 7, 13 and 30 days of culture in absence and in the presence of ascorbic acid. Values represent the average levels ± SEM of three separate experiments. $^{\circ}p<0.07$; $^{\circ\circ}p<0.03$ and $^{\circ\circ\circ}p<0.01$ when untreated cultures were compared with treated cultures.

control and DPPA sponges. With 10^6 cells per sponge (Fig. 1), chondrocyte numbers consistently increased with time reaching, in the absence of ascorbic acid and after 4 weeks in culture in the control and cross-linked sponges, 3.5×10^6 and 4.9×10^6 cells respectively. In contrast, in the presence of ascorbic acid, almost 2-fold increases in cell number were observed, reaching 6.5×10^6 and 8.5×10^6 cells, respectively. With 10^7 cells per sponge, after an initial decrease, cell numbers remained constant throughout culture, again with higher values in the presence of ascorbate ($12–15 \times 10^6$ cells in cross-linked sponges).

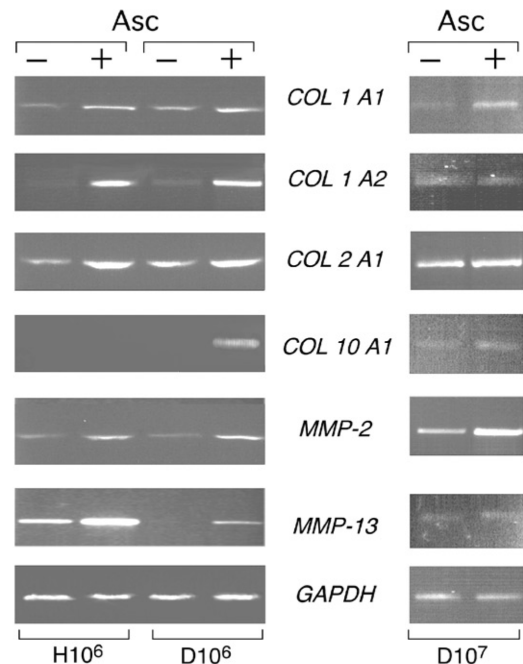

Fig. 2. Expression of selected genes by bovine chondrocytes grown in collagen sponges with (asc +) and without (asc −) ascorbic acid present throughout the culture. After 1 month in culture, RNAs were extracted from control (H) and cross-linked DPPA (D) sponges, initially seeded with 10^6 and 10^7 cells, followed by RT-PCR as described under Materials and Methods.

3.2. Analysis of matrix components

3.2.1. Chondrocyte gene expression in the presence or absence of ascorbic acid

The effects of ascorbic acid in the culture medium using sponges seeded with 10^6 and 10^7 cells were examined on day 30 by comparing relative gene

transcript levels in chondrocytes grown in treated or untreated cultures. As shown in Fig. 2, on the whole, increases were noted in the expression of all genes studied (types II, I (α1 and α2 chains), X collagens and MMPs-2 and -13) as a result of ascorbic acid treatment for both types of scaffold and for sponges seeded with 10^6 and 10^7 cells. In particular, the greatest increase in gene expression as a result of ascorbic acid treatment was observed with the sponges seeded with 10^6 cells for type I (α2 chain) collagen and MMPs-2 and -13. Type X collagen mRNA expression was detected in the DPPA-treated sponges (after 40 PCR cycles) in the presence of ascorbic acid.

3.2.2. Collagen synthesis and immunolocalisation

In order to confirm the data obtained at the gene level, we measured neosynthesis of total collagen as well as individual types I and II collagens, expressed per μg of DNA and either deposited in the sponge or secreted into the culture medium, on days 7 and 30 of culture after labelling for 14 h with ^{35}S methionine (Fig. 3). Whatever the culture conditions used (both types of support, under both seeding conditions and with or without ascorbate) collagen synthesis levels (per cell) and the percentage of neosynthesised collagen deposited in the sponge (75–85% of the total) showed only slight and insignificant variations with the exception of a net decrease in the collagen synthesised per cell after 30 days in sponges seeded with 10^6 cells in the presence of ascorbate (Fig. 3 B1).

The main differences observed between cultures with or without ascorbate concerned the neosynthesised type I/type II collagen ratio. In the absence of ascorbate (Fig. 3 C1) small amounts of type I collagen (5–15% of total) were observed mostly after 30 days and especially in the culture medium. In the presence of ascorbate, these values reached 10–35% of the collagen deposited in the sponge (Fig. 3 D1) and 30–45% of the collagen secreted into the medium (Fig. 3 D2). Immunohistology with antibodies against types II and I collagens (Fig. 4) demonstrated that type II collagen was located mostly where cells were forming aggregates in both types of scaffold and was more abundant (even in the presence of ascorbate (Fig. 4a)) than type I collagen, present either in small patches throughout the sponges (Fig. 4b) or at the periphery of the scaffold. It is interesting to note that the initial scaffold, still clearly detectable after 1 month culture, was not recognised by the antibodies against native type I collagen.

3.2.3. GAG content

Sulphated glycosaminoglycan (sGAG) content was similar in both control and cross-linked sponges and was dependent on initial cell density (Fig. 5). After 30 days in culture in absence of ascorbic acid, the amounts of sGAG in both types of sponge reached a maximum of 0.9 mg in sponges initially seeded with 10^7 cells and only 0.15 mg in sponges initially seeded with 10^6 cells. This low deposition of sGAG in the latter case, was not modified by the addition of ascorbic acid, whereas after 1 month in culture a slight increase in sGAG was shown in native and cross-linked scaffolds for sponges seeded with 10^7 cells (+31 and +17% respectively) as compared with untreated cultures. The net difference in sGAG content in the sponges seeded with 10^6 and 10^7 cells was clearly demonstrated by histology using Safranin O staining (Fig. 4c and d).

3.3. Metalloproteinase analysis

To investigate the involvement of MMPs in neomatrix production, the culture media were analysed for gelatinase activity (MMP-2 and-9) by gelatin substrate zymography (Fig. 6A). Traces of proMMP-9 were only detected for cultures in native sponges. Most of the MMP-2 was present as inactive forms in spent media and the total content of the pro- and active forms increased with time to reach values that were not significantly different either without ascorbic acid (Fig. 6 B1) or with (Fig. 6 B2). However, a higher percentage of an active form of MMP-2) was noted after 30 days in the presence of ascorbic acid (Fig. 6 C2) than in its absence (Fig. 6 C1) (12–18% instead of 8–10% respectively). MMP-13 activity was not detected in the medium tested for MMP-2 even after a 10-fold concentration of the medium.

4. Discussion

Many physiological and biochemical processes appear to be influenced by ascorbate [77], this being an essential requirement in normal connective tissue metabolism and collagen formation. As a reducing agent, ascorbate is a cofactor for prolyl and lysyl hydroxylase and its absence prevents the efficient secretion of procollagen. Furthermore, ascorbate is also thought to enhance the transcription of procollagen mRNA in fibroblasts [78] without changing the rate of intracellular degradation. Ascorbate, in the form of stable derivatives such as vitamin C-phosphate, was also shown to improve cell proliferation when added in culture of rabbit renal proximal tubular cells [79], in combination with dexamethasone in culture of human anterior cruciate ligament cells [80] or in combination with bFGF in culture of human vascular myofibroblasts in PGA-P4HB coated scaffold [81]. Together with several others, our group has examined the effects of ascorbate on growth and synthesis of matrix components by cultured chondrocytes of different origins when plated both at low [82–87] and high [71,88,89] cell densities and as explants [90]. In addition, chondrocyte culture in a 3D

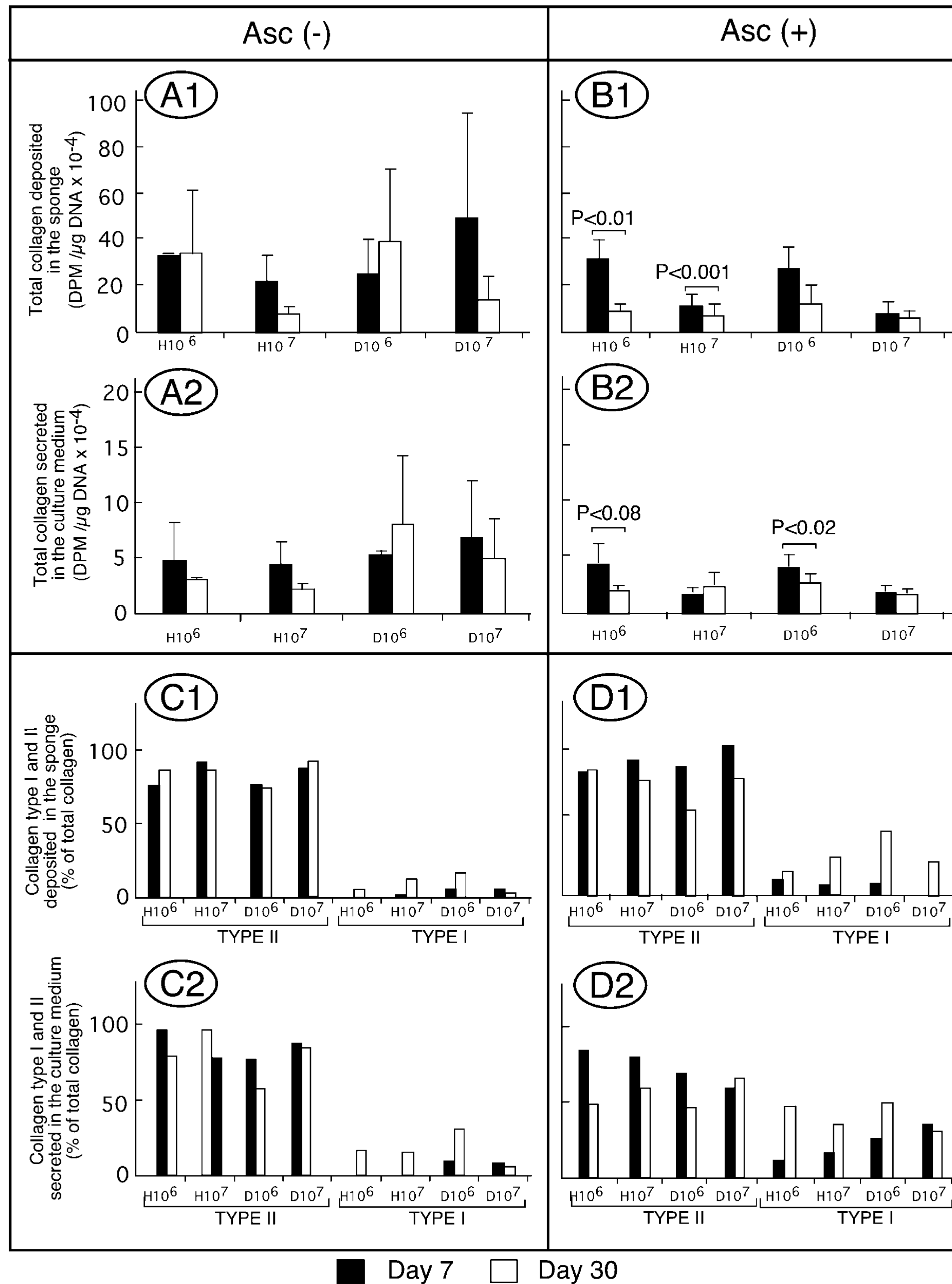

Fig. 3. Effect of ascorbic acid on the levels of collagens neosynthesised by bovine chondrocytes grown in collagen sponges and incubated with ^{35}S methionine for the last 14 h of culture on days 7 and day 30. Neosynthesised collagen (expressed in dpm/µg DNA $\times 10^{-4}$) deposited in the sponges in the absence of ascorbic acid (A1) and in its presence (B1). Neosynthesised collagen secreted into the culture medium in the absence of ascorbic acid (A2) and in its presence (B2). Levels of types II and I collagen (expressed as % of collagen types XI, II and I separated on SDS-PAGE and quantified as described in Materials and Methods) deposited in the sponges in the absence of ascorbic acid (C1) and in its presence (D1). Levels of types II and I collagen secreted into the culture medium in the absence of ascorbic acid (C2) and in its presence (D2).

environment (within natural or synthetic matrices) is now an active domain of research in the preparation of cell-seeded matrices for use as implants to facilitate cartilage repair. Among the bioactive factors thought to be involved in the generation of cartilaginous constructs in vitro several growth factors (PDGF, EGF, TGF-b, FGF, IGF) have been tested [15,24,40,61,91,92], while the specific influence of ascorbate has not yet been investigated in detail, even though most of the culture medium usually contains ascorbic acid.

We have thus completed our previous study [49] on the growth and metabolism of fetal epiphyseal chondrocytes cultured within collagen sponges but carried out without the addition of fresh ascorbic acid. Several

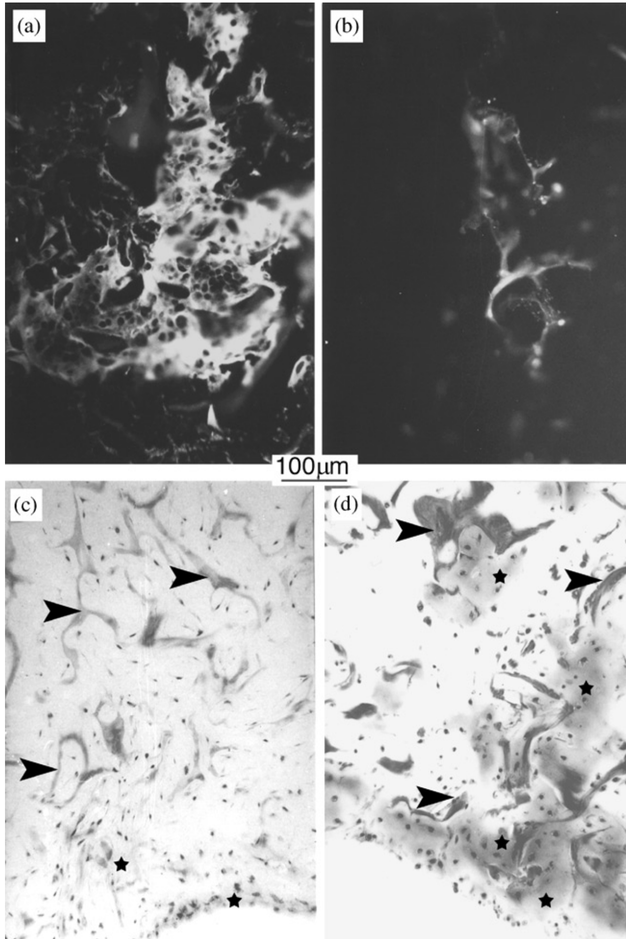

Fig. 4. Immunohistology of collagen types II and I and glycosaminoglycan staining of native collagen sponges initially seeded with fetal bovine chondrocytes and grown for 1 month with ascorbic acid. (a) immunolabelling with antibodies against type II collagen in sponges initially seeded with 10^7 cells ($\times 150$); (b) immunolabelling with antibodies against type I collagen in sponges initially seeded with 10^7 cells ($\times 150$). Glycosaminoglycan staining with safranin O, in sponges initially seeded with (c) 10^6 cells and (d) 10^7 cells ($\times 150$). Bar = 100 μm. Arrow heads indicate the sponge and stars the deposition of GAG.

interesting results have been obtained regarding these parameters in this study. First, the addition of ascorbate induced a net increase in cell proliferation (almost a 2-fold increase after 1 month culture when compared to culture without ascorbate), independent of the initial cell-seeding density and of the nature of the collagen sponge (cross-linked or not). As previously observed [49] chondrocyte growth rate was dependent on initial cell-seeding density. With a low initial cell-seeding density (2.3×10^6 cells/cm^3) and after 1 month culture, we observed an up to 8.5-fold increase in cell numbers within the sponge. This result can be compared to growth rates obtained by different authors for chondrocytes cultured at similar low initial cell-seeding densities ($0.5–2 \times 10^6$ cells/cm^3) and in the presence of

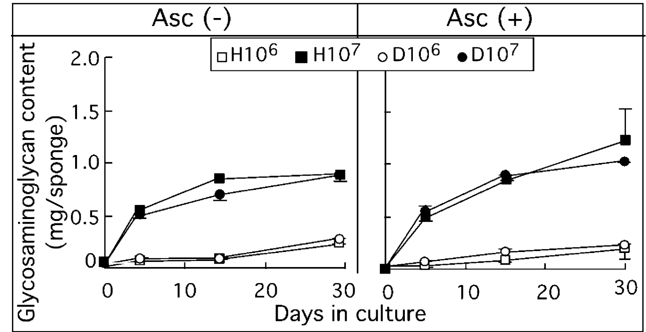

Fig. 5. Effect of ascorbic acid added throughout the culture on the total sulphated glycosaminoglycan (sGAG) content in collagen sponges. Cells were seeded in either control (H) or in DPPA (D) sponges at 10^6 and 10^7 cells per sponge. sGAG content was measured by a colorimetric method with dimethylmethylene blue. Data are presented as the mean ± SD of triplicate samples.

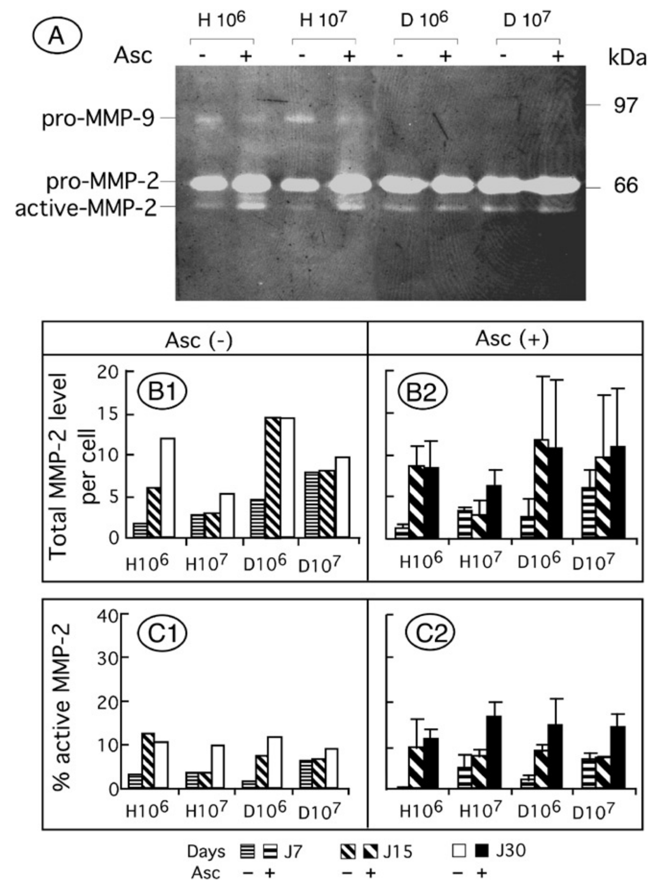

Fig. 6. Effect of the presence of ascorbic acid throughout the culture on the secretion of MMPs-2 and -9 into the medium by bovine chondrocytes grown in collagen sponges. Cells were seeded at 10^6 and 10^7 per control (H) and DPPA (D) sponges. The sponges were transferred to serum-free medium 48 h before harvesting the conditioned media samples at days 7, 15 and 30. (A) Culture media, harvested on day 30, subjected to gelatin zymography. (B) Histograms showing expression levels in arbitrary units (intensity per cell) of total MMP-2 (pro-MMP-2 and active MMP-2) in culture in the absence (B1) or presence (B2) of ascorbic acid. (C) Histograms showing the percent active MMP-2 compared with total MMP-2 in the culture medium in the absence (C1) or presence (C2) of ascorbic acid.

ascorbate but in different 3D environments: 5-fold and 2.25-fold increases with rabbit [43] and human [45] chondrocytes, respectively, after 1 month culture in collagen gels, a 2.2-fold increase with bovine chondrocytes after 57 days in an alginate disk [22], and a 2.2-fold with sheep articular chondrocytes after 9 weeks in a poly (L/DL-lactide) porous scaffold [60]. With a high initial cell-seeding density (2.3×10^7 cells/cm^3) cell losses were observed at the beginning of the culture followed by limited cell growth (maximum 1.5-fold increase after 1 month with ascorbate) as previously noted by several authors using collagen-GAG [42], polyglycolic acid [52] or fibrin [15] scaffolds and similar or higher initial cell-seeding densities. These culture conditions gave proliferation levels similar to those we obtained previously with the same chondrocytes plated at a high cell density (0.8×10^6 cells/cm^2) on plastic [71].

Second, metabolic activities of the chondrocytes were compared, with or without ascorbate, by analysing the expression and synthesis of different collagens and MMPs. With ascorbate, we observed an increase in the expression of genes encoding collagen types I, II and X and MMPs-2 and 13 after normalisation to a reference mRNA (GAPDH) but non-significant increases in global collagen synthesis (dpm/mg DNA) and sulphated glycosaminoglycan content of the sponges. After metabolic labelling of the cultures with ^{35}S methionine, pepsin treatment and SDS-PAGE, we confirmed the relative increase with ascorbate of type I collagen synthesis with a 2-3-fold increase in the ratio of neosynthesised collagen types I to II. Even though type II collagen remained the main collagen synthesised and present in the sponge (as shown by immunohistology) after 1 month culture with ascorbate, the high amounts of type I collagen produced (up to 35% of total collagen deposited in the sponge and up to 45% of total collagen secreted in the medium) demonstrate the induction of a partial loss of the cartilaginous phenotype by ascorbate. Furthermore, as traces of type X collagen mRNA were detected in cross-linked sponges after 1 month with ascorbate, we confirm here that the gene expression of chondrocytes cultured in a 3D environment may be significantly influenced by ascorbate as previously shown with the same cells [71] or with avian, mouse or bovine chondrocytes [84–89] plated at low or high cell densities on plastic. This ascorbate induced maturation was not accompanied by increased synthesis of total collagen and proteoglycan and deposition of a more abundant extracellular matrix within the collagen sponges. It was previously suggested that ascorbate modulation of chondrocyte gene expression was independent of its role in collagen secretion [85].

Matrix metalloproteinases (MMPs) play important roles in the turnover of matrix components in normal cartilage. As several data support the hypothesis whereby matrix degradation in degenerative articular cartilage could be promoted by an imbalance between the levels of MMPs and their inhibitors, we analysed the influence of ascorbate on gene expression and synthesis of MMPs-2, -9 and -13 by chondrocytes in our 3D culture conditions. Ascorbate induced a net increase in both MMP-2 and MMP-13 mRNA, but at the protein level, only MMP-2 was detected by gelatin zymography with an increase in its active form (12–18% of total) whereas MMP-13 activity was undetectable. Parallel induction by ascorbate of collagen X and MMP-2 and -13 mRNA is consistent with a previous report [93] that mature hypertrophic chondrocytes are characterised by high MMP-2 and MMP-13 mRNA. However, although MMP-2 can activate pro-MMP-13 [94], its low levels as an active form under our culture conditions, even in the presence of ascorbate, could explain the absence of MMP-13 as an active enzyme.

In conclusion, evidence is provided of an important role for ascorbic acid in the regulation of chondrocyte development and matrix formation during culture in porous 3D collagen sponges. Under these conditions ascorbate stimulates chondrocyte proliferation but does not increase cartilage matrix synthesis (at least after 1 month culture) and progressively alters the phenotypic expression of these cells. Thus further studies to evaluate the potential of various scaffolds and culture conditions (for example the effects of growth factors and/or mechanical stimuli) in preparing functional cartilage implants should take into account, contrary to most recent studies, the presence or absence of ascorbic acid in the culture medium as an important biological parameter.

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