Phenotypic expression of equine articular chondrocytes grown in three-dimensional cultures supplemented with supraphysiologic concentrations of insulin-like growth factor-I

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Objective—To assess the effects of supraphysiologic concentrations of insulin-like growth factor-I (IGF-1) on morphologic and phenotypic responses of chondrocytes.

Sample Population—Articular cartilage obtained from 2 young horses.

Procedure—Chondrocytes were suspended in fibrin cultures and supplemented with 25, 12.5, or 0 mg of IGF-1/ml of fibrin. Chondrocyte morphology and phenotypic expression were assessed histologically, using H&E and Alcian blue stains, immunoreaction to collagen type I and II, and in situ hybridization. Proteoglycan content, synthesis, and monomer size were analyzed. The DNA content was determined by bisbenzimide-fluorometric assay, and elution of IGF-1 into medium was determined by IGF-1 radioimmunoassay.

Results—Both 12.5 and 25 µg of IGF-1/ml enhanced phenotypic expression of chondrocytes without inducing detrimental cellular or metabolic effects. Highest concentration of IGF-1 (25 µg/ml) significantly increased total DNA content, glycosaminoglycan (GAG) content, GAG synthesis, and size of proteoglycan monomers produced, compared with cultures supplemented with 12.5 µg of IGF-1/ml or untreated cultures. Histologic examination confirmed these biochemical effects. Matrix metachromasia, type-II collagen in situ hybridization and immunoreaction were increased in cultures treated with 25 µg of IGF-1/ml, compared with cultures supplemented with 12.5 µg of IGF-1/ml or untreated cultures.

Conclusions and Clinical Relevance—Chondrocytes exposed to high concentrations of IGF-1 maintained differentiated chondrocyte morphology and had enhanced synthesis of matrix molecules without inducing apparent detrimental effects on chondrocyte metabolism. These results suggest that application of such composites for in vivo use during cartilage grafting procedures should provide an anabolic effect on the grafted cells. (*Am J Vet Res* 2002;63:301–305)

Articular cartilage functions to provide pain-free nearly frictionless articular movements and resilience to applied biomechanical forces.[1] The response of cartilage to injury differs from that of other tissues because of the limited capacity of chondrocytes to proliferate, migrate to the site of injury, or up-regulate their synthetic capacities. The tissue formed during repair of full-thickness cartilage defects is typically fibrocartilaginous in composition and not able to withstand applied loads.[2-5] This inadequate intrinsic healing response has generated interest in the application of grafting procedures and growth factors in an attempt to enhance cartilage healing.

Insulin-like growth factor-1 (IGF-1) can stimulate chondrocyte metabolism in vitro.[6-9] Addition of exogenous IGF-1 to explant and monolayer cultures at concentrations of 10 to 200 ng/ml of medium resulted in increased synthesis of large aggregating proteoglycans and type-II collagen, inhibition of proteoglycan degradation, protection from the effects of matrix degrading cytokines, and, in some studies, increased synthesis of DNA. Ranges of IGF-1 concentrations up to 200 ng/ml extend beyond the physiologic concentrations of articular environments. Luyten et al[10] reported that the concentration of IGF-1 in articular cartilage from young calves is 50 ng/g, whereas Schneiderman et al[11] reported that the concentration of IGF-1 in adult human cartilage was only 3 to 9 ng/g with concentrations of 30 to 50 ng/ml in synovial fluid. The effects of high concentrations of IGF-1 on cartilage metabolism have been documented.[12] When up to 2 µg of IGF-1/ml of medium were used in explant studies, matrix-enhancing results similar to those in the aforementioned in vitro studies were observed, and there were no apparent deleterious effects.

Several investigators have described augmented cartilage repair by using various matrices, including fibrin.[13-18] Fibrin is a useful biological vehicle for chondrocyte transfer[19] and an effective depot for growth-factor supplementation of chondrocyte grafts.[20,21] An in vitro study[22] revealed that 25 µg of IGF-1 incorporated into 1 ml of polymerized fibrin eluted at concentrations ≥ 100 ng/ml for 22 days. However, the effects of such initial supraphysiologic concentrations (25 µg) of IGF-1 on chondrocyte viability or metabolism in fibrin, or the possibility of toxic effects, have not been determined. Information concerning the in vitro efficacy and effect of supraphysiologic concentrations of IGF-1 on phenotypic expression of chondrocytes are important for providing guidance regarding in vivo applications.

The objectives of the study reported here were to determine the morphologic and phenotypic responses of chondrocytes cultured in fibrin matrices supplemented with high concentrations of IGF-1. We chose

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to investigate the use of 12.5 and 25 µg of IGF-1/ml of fibrin-chondrocyte culture, because elution patterns suggest that chondrocytes in the fibrin matrix would be supplemented with a greater-than-physiologic concentration (> 100 ng/ml) for at least 22 days.[22] Our hypothesis was that high concentrations of IGF-1 would increase synthesis of matrix molecules but would not incite toxic events such as cell death, alteration of chondrocyte morphology, or a decrease in the synthesis of proteoglycans or type-II collagen.

Materials and Methods

Isolation of chondrocytes—Articular cartilage was harvested from the femoropatellar and femorotibial joints of 2 horses (4 and 8 months old, respectively). Chondrocytes were isolated from the matrix by digestion in 0.075% collagenase, as described elsewhere.[23] The medium used consisted of Ham's F-12 medium containing 50 µg of ascorbic acid/ml, 30 µg of α-ketoglutaric acid/ml, 300 µg of L-glutamine/ml, 10% fetal calf serum, 100 U of sodium penicillin/ml, and 100 µg of streptomycin sulfate/ml. Following isolation, a cell count and test for viability were performed, using the supra vital dyes fluorescein diacetate and propidium iodide.[24] Prior to implantation in fibrin, chondrocytes were cultured in monolayers to allow cell adhesion and to ensure that only viable chondrocytes were used for culture in fibrin. Monolayers were maintained for 2 days at 37 C, 5% CO_2, and 90% humidity. Monolayers then were digested in trypsin. Chondrocytes were pooled, centrifuged, and resuspended in media, and another cell count was performed.

Three-dimensional culture—Chondrocytes were allotted to 3 experimental groups (6.0×10^7 viable cells/group) and centrifuged again. Each group of pelleted chondrocytes was resuspended in 0.5 ml of lyophilized equine fraction-1 fibrinogen that was solubilized in medium to a final concentration of 200 mg/ml. The chondrocyte-fibrinogen mixture was drawn into a 3-0-ml syringe, and calcium-activated equine thrombin (200 U/ml) was drawn into another 3-ml syringe. Using a Y-connector, 0.5 ml of each component was combined during injection into wells of 24-well plates to yield 1-ml fibrin disks, each containing 10×10^6 chondrocytes. Three experimental groups, each consisting of 6 replicate wells, were created by adding IGF-1[a] at concentrations of 0 (control culture), 12.5, or 25 µg/ml of fibrinogen after polymerization. Disks were covered with medium and cultured for 14 days. Medium was exchanged on alternate days, and exhausted medium was stored in 10% (wt:vol) protease inhibitors (ε-amino-*n*-caproic acid [0.1*M*], benzamidine HCL [10 m*M*], pepstatin A [1 µ*M*], phenylmethylsulfonyl fluoride [10 m*M*], and Na_2EDTA [10 m*M*]) at -70 C.

Culture harvest—Sixteen hours prior to harvest, a 5-mm central slice of each disk was removed and fixed in 4% paraformaldehyde for routine histologic examination, type-I and -II procollagen in situ hybridization, and type-II collagen immunohistochemical analysis. The remaining portion of each disk was cultured for another 16 hours after addition of 20 µCi of [^{35}S]sulfate/ml. At harvest, half of each radiolabeled disk was rinsed in protease inhibitors, lyophilized, weighed, digested in 0.5% papain at 65 C, and assayed for total **glycosaminoglycan (GAG)**, [^{35}S]-GAG, and DNA content. The remaining half of each radiolabeled disk was incubated overnight in 4*M* guanidine hydrochloride, pH 6.0, to extract proteoglycan monomers for agarose size-exclusion chromatography.

Histologic examination—Disks fixed for histochemical analysis, type-I and -II procollagen in situ hybridization, and immunocytochemical analysis were dehydrated in alcohol, embedded in paraffin, and sectioned at a thickness of 6 µm. Sections were stained with H&E to allow evaluation of morphologic characteristics and Alcian blue-nuclear fast red for assessment of proteoglycan distribution in the pericellular matrix.

In situ hybridization—Sections were prepared for in situ hybridization, using a modification of techniques described elsewhere.[25] Identification and generation of equine-specific type-II procollagen riboprobes have been described.[20] Briefly, riboprobes were generated from a 201-base pair cDNA encoding exons 1 to 7 of equine type-IIB procollagen mRNA that corresponded to positions 138 to 339 of the available equine coding sequence.[b] Riboprobes for type-1 procollagen were generated from a 254-base pair cDNA encoding exons 1 to 2 of the NH_2-propeptide region that corresponded to positions 1 to 254 of the equine coding sequence.[c] Antisense and sense [^{35}S]-UTP-labeled riboprobes were transcribed on *Eco*RI-linearized pGEM-3zf(+) DNA templates.[d] Following hybridization and washing, sections were dipped in an emulsion[e] and exposed for 14 days. The emulsion was developed,[f] and sections were counterstained with hematoxylin. Examinations of sections were conducted, using light- and dark-field microscopy.

Immunohistochemical analysis—Sections were processed as reported elsewhere,[20] using rat anti-bovine type-II collagen primary antibody[g] (1:100) or rabbit anti-equine type-I collagen primary antibody (1:10) and secondary antibody,[h] followed by streptavidin-conjugated peroxidase to catalyze chromogen development in 3,3'-diaminobenzidine tetrachloride. Sections were counterstained with Harris hematoxylin and examined by use of microscopy to determine distribution of type-I and -II collagen.

DNA analysis—Total DNA was determined on duplicate 100-µl aliquots of papain digests incubated for 24 hours at 65 C. Samples were mixed with 2.5 ml of 0.2 mg of bisbenzimide compound[i]/ml for DNA quantification by fluorometric assay.[26] Fluorescence was evaluated by spectrofluorometry with an excitation wavelength of 348 and emission measured at 456 nm. Calf thymus DNA was used to prepare a standard curve.

Total proteoglycan content—Total proteoglycan content of medium and disks was assayed by the **dimethyl-methylene blue (DMMB)** binding spectrophotometric assay, using 50 µl of papain-digested disk material or 500 µl of undigested media added to 2.5 ml of DMMB solution (in formate buffer), and the optical density was determined at 525 nm.[27] Mixed-isomer shark chondroitin sulfate was used to construct the standard curve. Incorporation of [^{35}S]sulfate into medium and disk-associated GAG was determined by Alcian blue precipitation of 25-µl aliquots in multiple-well punch plates[j] and scintillation counting.[28]

Hydrodynamic size of proteoglycan monomers—Unincorporated [^{35}S] was separated from the extracted proteoglycan monomers by use of 10,000-kd centrifuge columns.[k] Monomers were eluted under dissociative conditions (4*M* guanidine hydrochloride, pH 6.0) through a 105-cm agarose column.[l] Scintillation counting was performed on 300 µl of each 750-µl fraction to determine hydrodynamic size of proteoglycan molecules and partition coefficients of each experimental group.[21] Proteoglycans extracted from articular cartilage obtained from a clinically normal 1-year-old horse were used to determine the column void, and free [^{35}S] was used to determine the column total volume.

IGF-1 radioimmunoassay—Elution of IGF-1 from fibrin disks was determined by use of a competitive-binding radioimmunoassay[m] performed in accordance with the manufacturer's directions. Briefly, 100 µl of medium was incubat-

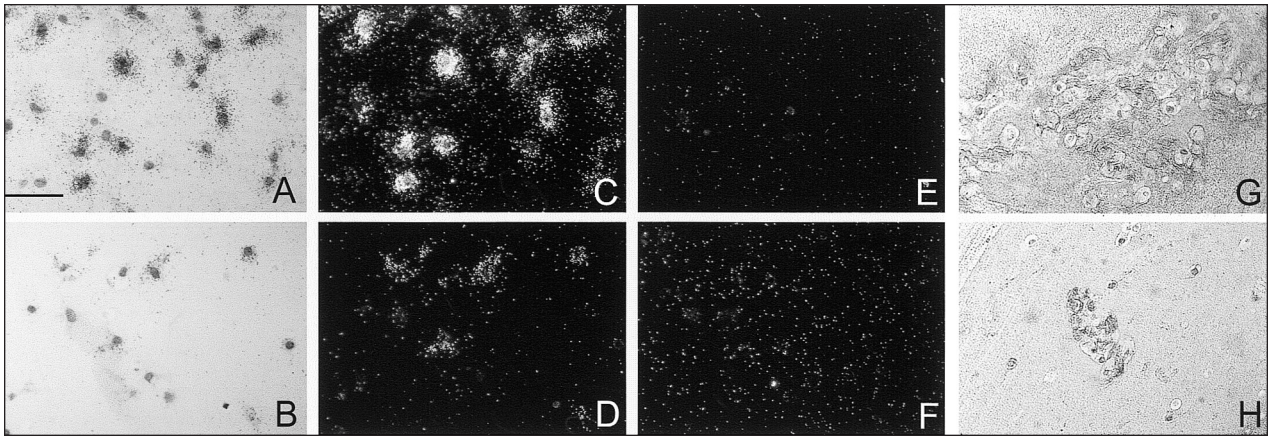


Figure 1—Photomicrographs of chondrocytes cultured for 14 days in fibrin supplemented with of 25 μg of insulin-like growth factor-I (IGF-1)/ml (A, C, E, G) or 12.5 μg of IGF-1/ml (B, D, F, H). Notice the greater hybridization to type-II procollagen and increased immunoreactivity in chondrocytes grown in cultures supplemented with 25 μg, compared with cultures supplemented with 12.5 μg. Panels A to D are results of in situ hybridization to type-II procollagen mRNA; panels A and B are bright-field microscopy and panels C and D are dark-field illumination of the same images. Panels E and F are sections hybridized to sense probes (control probes). Panels G and H represent results of immunohistochemical analysis for type-II collagen. Bar = 25 μm.

ed with 900 μl of acid-ethanol solution (12.5% 2N HCl:87.5% ethanol [vol:vol]) to dissociate IGF-1 from IGF-binding proteins. The resulting solution was incubated at 27 C for 30 minutes, which was followed by centrifugation to precipitate IGF-binding proteins. The pH of the supernatant was neutralized by adding 0.855*M* Tris-base, and a second centrifugation was performed to eliminate interference from additional precipitate. Rabbit anti-IGF-1 serum was added to the supernatant, and the mixture was allowed to incubate for 1 hour. This was followed by addition of [^{125}I]IGF-1 and incubation for an additional 16 hours. Precipitating complex (normal rabbit serum previously precipitated with goat anti-rabbit serum and polyethylene glycol) was added to each sample, which then was incubated for 20 minutes. Samples were centrifuged, and the amount of precipitated IGF-1 was determined by gamma scintillation counting and comparison to values obtained for a standard curve prepared from samples supplied by the manufacturer.

Statistical analysis—Differences in the mean total μg of GAG for each disk/μg of DNA, log ([^{35}S]-GAG **counts per minute [cpm]**/μg of DNA), μg of DNA/mg of dry weight, log ([^{35}S]-GAG cpm/ml of medium), and total μg of proteoglycan in the medium/ml of medium were evaluated with an ANOVA to compare values among the 3 treatment groups (0, 12.5, and 25 μg/ml) at day 14 of culture. When an ANOVA revealed a significant F-test, the Tukey post-hoc procedure was performed to determine the treatment groups that differed significantly from each other. Changes in medium content of proteoglycan over time were analyzed by use of linear regression. For all analyses, values of $P \leq 0.05$ were considered significant.

Results

Histologic examination—In all culture groups, chondrocytes maintained a rounded chondrocyte appearance without evidence of dedifferentiation, such as changing shape to a more fibroblastic appearance, chondrocyte clustering or cloning, or expression of type-I collagen mRNA or protein. Cultures supplemented with 25 μg of IGF-1 had increased metachromasia of the pericellular matrix, immunoreactivity to type-II collagen, and hybridization to type-II procollagen mRNA, compared with chondrocytes treated with 12.5 μg of IGF-1 or untreated (control) cultures (**Fig 1**). Histochemical differences were not detected between cultures supplemented with 12.5 μg of IGF-1 and control cultures.

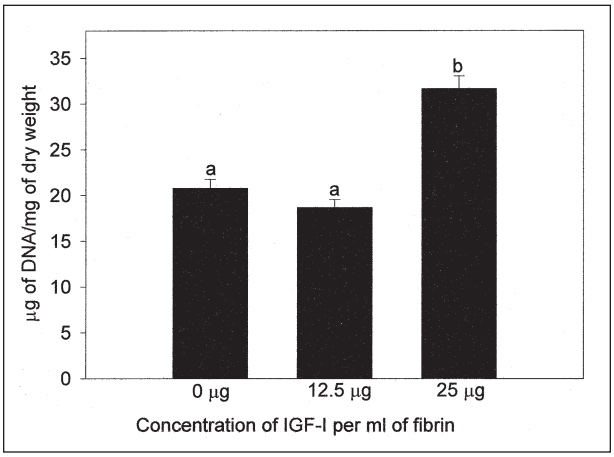
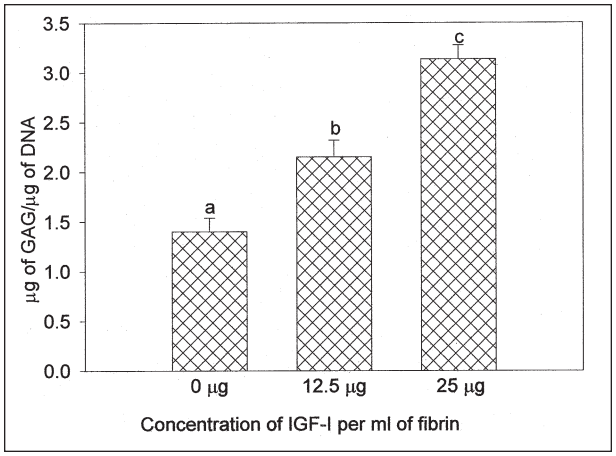


Figure 2—Total DNA content in chondrocytes cultured for 14 days in fibrin supplemented with varying concentrations of IGF-1. Values reported represent mean ± SEM for 6 cultures. [a,b]Values with different letters differ significantly ($P < 0.05$; Tukey post-hoc test).

DNA analysis—A significant increase in DNA content was detected in cultures supplemented with 25 μg of IGF-1, compared with cultures supplemented with 12.5 μg of IGF-1 and untreated cultures (**Fig 2**).

Proteoglycan analyses—Biochemical analyses supported the histologic observations of an increased accumulation of GAG (**Fig 3**) and [^{35}S]-incorporation into newly synthesized GAG in chondrocyte cultures supplemented with IGF-1. Maximal effects were evident in cultures containing 25 μg of IGF-1. Additionally, proteoglycan monomers were largest in cultures supplemented with 25 μg of IGF-1 (**Fig 4**). There was not a significant difference among treatment groups in the amount of GAG released into the medium over time (**Fig 5**).

Elution of IGF-1—Elution of IGF-1 from fibrin cultures containing 25 μg of IGF-1 decreased significantly between days 2, 4, 6, and 8 of culture and then remained



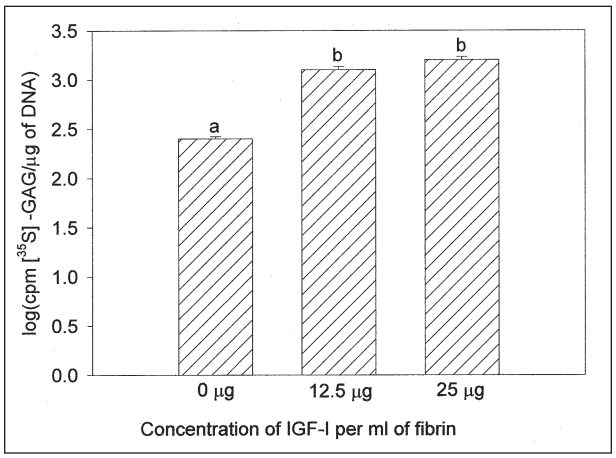


Figure 3—Total glycosaminoglycan (GAG) content (A) and ^{35}[S]-GAG synthesis (B) in chondrocytes cultured for 14 days in fibrin supplemented with varying concentrations of IGF-1. Values reported represent mean ± SEM for 6 cultures. [a,b,c]Values with different letters differ significantly ($P < 0.05$; Tukey post-hoc test).

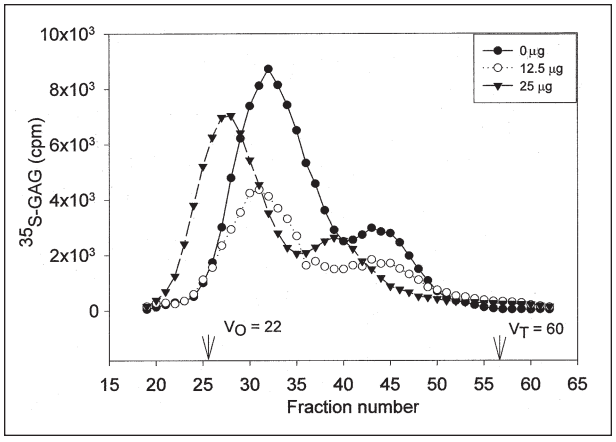


Figure 4—Effects of supplementation of culture media with IGF-1 on size of synthesized proteoglycan monomers determined by dissociative column chromatography and scintillation counting. Graph of each symbol represents a chromatogram for 6 samples pooled from each treatment group. Chondrocyte cultures supplemented with 25 μg of IGF-1 produced the largest proteoglycan monomers. cpm = Counts per minute. V_0 = Void volume. V_T = Total volume.

at > 50 ng/ml for the remainder of the 14-day study (**Fig 6**). Elution of IGF-1 from fibrin cultures containing 12.5 μg of IGF-1 followed a similar pattern, with IGF-1 eluting at lower concentrations. We did not detect significant differences in the concentration of IGF-1 in the medium of fibrin disks cultured without IGF-1 supplementation.

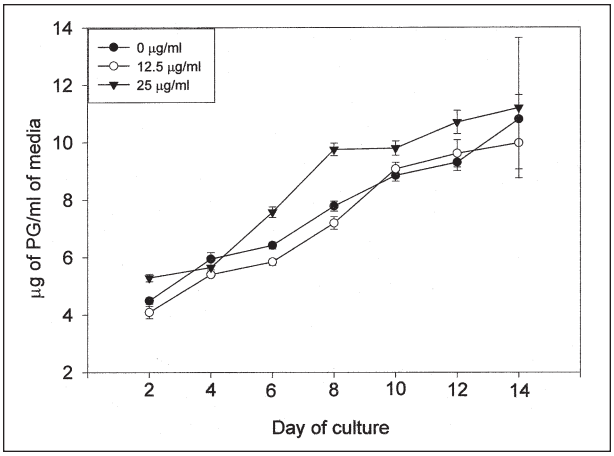


Figure 5—Total proteoglycan (PG) synthesized and released into culture medium supplemented with various concentrations of IGF-1 as a function of time. Values reported represent mean ± SEM for 6 cultures. Significant differences were not detected among treatment groups on the basis of analysis by use of linear regression.

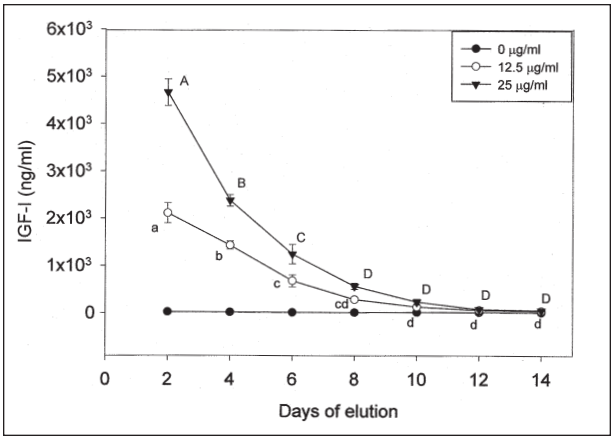


Figure 6—Concentration of IGF-1 in culture medium. Values reported represent mean ± SEM of 6 cultures. [a-e]Within cultures supplemented with 12.5 μg of IGF-1/ml, values with different letters differ significantly ($P < 0.05$; Tukey post-hoc test). [A-D]Within cultures supplemented with 25.0 μg of IGF-1/ml, values with different letters differ significantly ($P < 0.05$; Tukey post-hoc test).

Discussion

Chondrocyte proliferation and expression of cartilage-specific matrix proteins were maximally enhanced in fibrin cultures supplemented with 25 μg of IGF-1/ml. Histologic examination revealed increased matrix metachromasia, type-II collagen mRNA, and type-II collagen protein content in cultures treated with 25 μg of IGF-1/ml of fibrin, compared with values for cultures supplemented with 12.5 μg of IGF-1/ml or untreated cultures. This pattern of chondrocyte responsiveness to differing concentrations of IGF-1 was confirmed by results of biochemical analyses in which 25 μg of IGF-1/ml of fibrin stimulated increases in total DNA, GAG content and synthesis, and size of proteoglycan monomers to a greater extent than was evident in cultures supplemented with 12.5 μg of IGF-1/ml or untreated cultures.

Cultures supplemented with 12.5 and 25 µg of IGF-1/ml of fibrin had increased synthesis of matrix proteins, compared with untreated cultures. Initial concentrations of IGF-1 in medium from cultures supplemented with 25 µg/ml indicated that more than 4 µg/ml had eluted from the fibrin cultures during the first 48 hours. However, residual IGF-1 in the fibrin matrix exceeded 100 ng/ml for 8 days and resulted in enhanced phenotypic expression of chondrocytes. These data were consistent with the rate of IGF-1 loss from fibrin disks determined on the basis of results for IGF-1 elution. Analysis of elution data for the study reported here suggested a more rapid loss of IGF-1 from fibrin cultures containing chondrocytes than was reported[22] for cultures devoid of chondrocytes in which 25 µg of IGF-1 eluted at a rate of ≥ 100 ng/ml for 22 days. The IGF-1 elution pattern for untreated cultures also revealed that there was approximately 25 ng of IGF-1/ml of medium in cultures without IGF-1 supplementation, suggesting a measurable amount of IGF-1 in the 10% fetal bovine serum used for medium supplementation. Detection of a measurable IGF-1 concentration in untreated cultures may also have been the result of an autoinductive IGF-1 autocrine-paracrine effect, similar to that reported elsewhere.[29]

Analysis of these data suggested that fibrin laden with high concentrations of IGF-1 released IGF-1 to the surrounding chondrocytes at concentrations suitable to stimulate synthesis of matrix proteins, but more importantly, the high concentrations of IGF-1 initially contained within the fibrin cultures did not induce apparent detrimental effects on chondrocyte survival or metabolism. Application of high concentrations of IGF-1 in fibrin-chondrocyte graft composites for in vivo repair of articular defects in animals would seem appropriate on the basis of a lack of detrimental effects in vitro.

[a]IGF-1, Genentech Inc, San Francisco, Calif.
[b]GenBank accession No. U62528.
[c]GenBank accession No. AF034691.
[d]pGEM-3zf(+) plasmid DNA, Promega Corp, Madison, Wis.
[e]NTB2 emulsion, Eastman Kodak Co, Rochester, NY.
[f]D-19, Eastman Kodak Co, Rochester, NY.
[g]Provided by Dr. Michael Cremer, Department of Anatomy, Veterans Administration Hospital, Memphis, Tenn.
[h]Supersensitive multilink, BioGenex, San Ramone, Calif.
[i]bisbenzimide compound, Sigma Chemical Co, St Louis, Mo.
[j]PDVF multiwell punch plate, Millipore Intertech, Bedford, Mass.
[k]Centricon Concentrators, Amicon, Beverly, Mass.
[l]Sepharose CL2B, Pharmacia Biotech AB, Uppsala, Sweden.
[m]Nichols Institute Diagnostics, San Juan Capistrano, Calif.

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