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Increased Matrix Synthesis Following Adenoviral Transfer of a Transforming Growth Factor β_1 Gene into Articular Chondrocytes

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Summary: Monolayer cultures of lapine articular chondrocytes were transduced with first-generation adenoviral vectors carrying lacZ or transforming growth factor β_1 genes under the transcriptional control of the human cytomegalovirus early promoter. High concentrations of transforming growth factor β_1 were produced by chondrocytes following transfer of the transforming growth factor β_1 gene but not the lacZ gene. Transduced chondrocytes responded to the elevated endogenous production of transforming growth factor β_1 by increasing their synthesis of proteoglycan, collagen, and noncollagenous proteins in a dose-dependent fashion. The increases in collagen synthesis were not accompanied by alterations in the collagen phenotype; type-II collagen remained the predominant collagen. Transforming growth factor β_1 could not, however, rescue the collagen phenotype of cells that had undergone phenotypic modulation as a result of serial passaging. These data demonstrate that chondrocytes can be genetically manipulated to produce and respond to the potentially therapeutic cytokine transforming growth factor β_1 . This technology has a number of experimental and therapeutic applications, including those related to the study and treatment of arthritis and cartilage repair.

Articular cartilage has a limited capacity to repair damage sustained as a result of disease or injury (25). Although there are a variety of surgical and pharmacological approaches to enhancing cartilage repair, none is ideal (25). Improvements in understanding the biology of articular chondrocytes have provided opportunities for improving the healing of articular cartilage by biological means. An attractive example of this approach is to employ cytokines that increase matrix synthesis, decrease matrix breakdown, or do both (8,25,31).

Although such molecules hold much promise in this regard, their clinical application is likely to be limited by problems associated with the need for maintaining high concentrations of these cytokines at sites of cartilage damage for sustained periods of time. Direct application of the substances themselves may not be useful because of their short biological half-lives. For example, it was recently reported that the half-life of bone morphogenetic protein-2 (BMP-2) introduced

into an experimental lesion in the articular cartilage of rabbit knee joints is 4 days (14). One approach to solving this sort of problem involves the implantation of a matrix that releases the factor at biologically relevant concentrations. Gene transfer offers an alternative approach (9).

Cytokines are proteins, and there are several potential advantages to delivering to cartilage the genes encoding therapeutic cytokines rather than the cytokines themselves (9). One advantage is that the reparative factors are synthesized locally in a sustained and potentially regulatable fashion at the site of injury. And, unlike recombinant factors produced by bacteria, those synthesized endogenously as a result of gene transfer have undergone authentic post-translational modification and may therefore have improved potencies. The feasibility of transferring genes to experimental lesions within articular cartilage has already been demonstrated (5,20).

Transforming growth factor β_1 (TGF- β_1) is a powerful modulator of cartilage metabolism, which can promote matrix synthesis, inhibit matrix catabolism, and alter chondrocyte proliferation rates *in vitro* and *in vivo* (18,23,24,26,28,29,34). Moreover, TGF- β_1 can counteract the suppressive effects of inflammatory

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mediators such as interleukin-1 (IL-1) on proteoglycan synthesis in cartilage (32,34). TGF- β_1 stimulates replenishment of proteoglycans in depleted cartilage *in vivo* (34), and osteoarthritic cartilage is more sensitive to TGF- β stimulation than is normal healthy cartilage (21). Thus, TGF- β_1 may be well suited for use in the repair of cartilaginous lesions resulting from arthritis or injury. Despite the beneficial effects of TGF- β on cartilage, however, its effects on synovium are deleterious. As a result, the direct introduction of TGF- β_1 protein or genes (1,12,13,33,34) into joints can be highly inflammatory and fibrotic, leading to cartilage degeneration and osteophyte formation. In view of these properties, it is necessary to deliver TGF- β intracartilagiously under conditions in which the synovium is not exposed to this cytokine. The present study therefore focuses on delivering the gene for TGF- β_1 to articular chondrocytes with use of an adenoviral gene transfer system.

MATERIALS AND METHODS

Cell Isolation and Transduction

Articular cartilage was recovered from the knee and shoulder joints of young adult (approximately 6 months old) New Zealand White rabbits and diced into small pieces (15). Chondrocytes were

adenovirus or treated with human TGF- β_1 protein purified from platelets (R and D Systems, Minneapolis, MN, U.S.A.). First-generation adenoviruses ($\Delta E1$ and $\Delta E3$) containing a human TGF- β_1 gene or, as a control, the lacZ gene, under the transcriptional control of human cytomegalovirus early promoter were generated through Cre-lox recombination (16) and propagated in 293 cells (American Type Culture Collection [ATCC], Rockville, MD, U.S.A.). The virus containing the lacZ gene was used to assess the susceptibility of the cells to adenoviral transduction and as a control for the effects of the TGF- β_1 gene.

Transduction of cells with adenovirus containing the lacZ gene (AdlacZ) or cDNA encoding human TGF- β_1 (AdTGF- β_1) was performed in 300 μ l of Gey's balanced salt solution for 1 hour at 37°C at various multiplicities of infection. Following transduction, the remaining supernatant was removed and replaced with 1 ml of serum-free Neuman-Tytell medium (GIBCO BRL, Grand Island, NY, U.S.A.) supplemented with antibiotics or Ham's F₁₂ medium supplemented with 10% fetal bovine serum and antibiotics (GIBCO BRL). Cells serving as untransduced controls were treated in the same manner as adenovirally transduced cells. To assess the expression of β -galactosidase, the product of the lacZ gene, cells were stained with 0.1% X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactose; Sigma Chemical, St. Louis, MO, U.S.A.) 48 hours after transduction by standard methods. The percentage of the cells expressing lacZ was determined by visual inspection. Nontransduced confluent cells were treated with purified TGF- β_1 protein in serum-less Neuman-Tytell medium. Following the transduction of cells or the addition of purified protein to serum-less medium, the cells were incubated for 24 hours before the addition of radionuclides used to determine the synthesis of matrix compo-

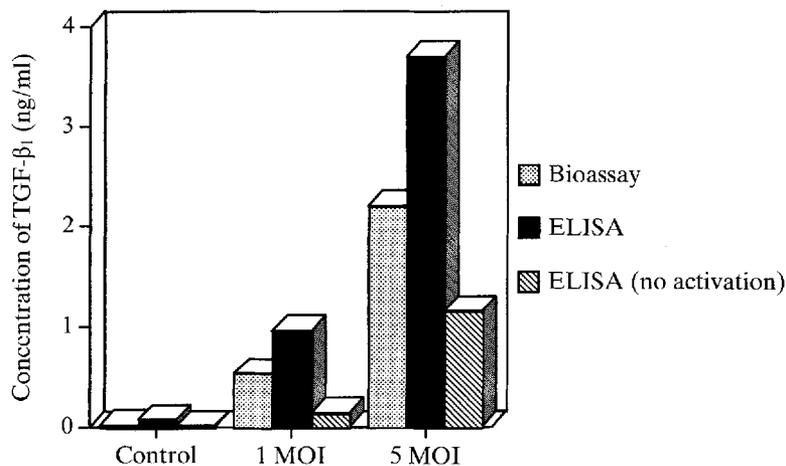


FIG. 1. Production of transforming growth factor β_1 (TGF- β_1) by chondrocytes. Supernatant from control cells and cells transduced with increasing amounts of adenovirus containing cDNA encoding human TGF- β_1 (AdTGF- β_1) were recovered. Latent TGF- β_1 was activated by heating. Active TGF- β_1 was assayed by enzyme-linked immunosorbent assay (ELISA) and bioassay. In all assays, untransduced control cells produced negligible amounts of TGF- β_1 . Supernatants from cells transduced with adenovirus containing the lacZ gene (AdlacZ) also produced negligible amounts of TGF- β_1 (data not shown). MOI = multiplicity of infection.

released by the sequential digestion of the cartilage fragments with trypsin (2% wt/vol; 30 minutes) and collagenase (2% wt/vol; 3 hours). The chondrocytes were seeded into monolayer culture at a density of 10^5 cells/cm² on 24-well plates with use of Ham's F₁₂ nutrient medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. For most experiments, cells were used without passaging. In those experiments in which the effects of passaging were evaluated, confluent monolayers were trypsinized (0.2% trypsin; 30 minutes), washed, and reseeded into nutrient medium at a 1:2 split ratio. This process was repeated to generate third-passage cells.

Two treatment groups comprised cells either transduced with

elements. Experiments were run in duplicate (i.e., $n = 2$ for each experiment). Each experiment was run at least twice (i.e., total $n = 4$) or three times (i.e., total $n = 6$).

Determination of TGF- β_1 Concentration

Latent TGF- β_1 was converted to active TGF- β_1 by heating the sample at 80°C for 15 minutes. TGF- β_1 concentrations in conditioned media were determined with a commercially available enzyme-linked immunosorbent assay (ELISA) kit and protocol (R and D Systems) and by the mink lung cell bioassay (19).

The ELISA protocol is a sandwich assay in which samples are added to 96-well plates coated with the type-II TGF- β soluble receptor. This captures any TGF- β_1 in the sample, which is detected

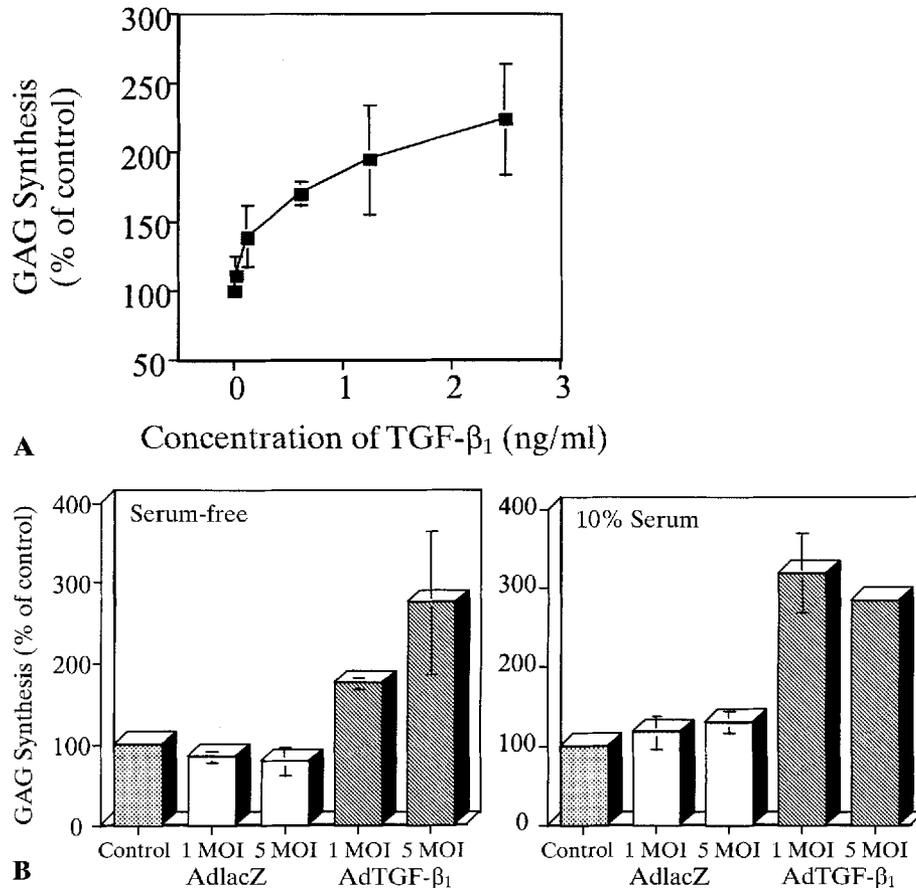


FIG. 2. Proteoglycan synthesis in response to transforming growth factor β_1 (TGF- β_1) (**A**) exogenously applied protein and (**B**) endogenously synthesized by transduced cells. **A:** Exogenous TGF- β_1 . Confluent chondrocytes were treated with different concentrations of purified, active TGF- β_1 for a total of 48 hours prior to determination of proteoglycan synthesis. The growth factor was replenished in the serum-free media after 24 hours. A dose-dependent increase in proteoglycan synthesis was obtained with increasing concentrations of TGF- β_1 . The individual data points are averages from triplicate experiments ($n = 6$) with results reported as mean \pm SD. **B:** Endogenous TGF- β_1 . Confluent chondrocytes were transduced with adenovirus containing the lacZ gene or cDNA encoding human TGF- β_1 (AdlacZ or AdTGF- β_1) in the concentrations indicated and subsequently maintained for 48 hours in serum-free media or media containing 10% fetal calf serum. Mean \pm SD of triplicate experiments ($n = 6$) are shown. GAG = glycosaminoglycan, and MOI = multiplicity of infection.

by an anti-TGF- β_1 antibody covalently linked to peroxidase. A colorimetric assay is used to measure the activity of the bound peroxidase, which is converted to TGF- β_1 concentration by a standard line. The assay fails to detect latent TGF- β_1 because the epitopes recognized by the enzyme-linked antibody are hidden by the latency-associated protein.

For the bioassay, bovine serum albumin (1 ng/ml) and 1 μ g/ml of each of the proteinase inhibitors aprotinin, leupeptin, and pepstatin were added to conditioned media. Mink lung epithelial cells (American Type Culture Collection [ATCC]) were seeded into 24-well plates with minimal Eagle medium supplemented with 10% fetal bovine serum, serum antibiotics, and glutamine. Six hours later, the medium was changed to one with only 0.5% fetal bovine serum and the culture continued for 18 hours. At this time, the chondrocyte-conditioned media or purified TGF- β_1 standards were added and incubation continued for an additional 6 hours. The cultures were then pulse-labeled with [3 H]thymidine for 2 hours, and the incorporation of radioactivity into material precipitated by trichloroacetic acid was measured by liquid scintillation.

Proteoglycan and Collagen Synthesis

To determine the effect of TGF- β_1 on matrix production, the synthesis of proteoglycan by adenovirally transduced chondro-

cytes and chondrocytes treated with exogenously added TGF- β_1 was measured. The synthesis of proteoglycans was measured as the incorporation of $^{35}\text{SO}_4^{2-}$ into glycosaminoglycans (30). $\text{Na}_2^{35}\text{S}\text{O}_4^{2-}$ (40 μCi [$1,480 \times 10^3$ Bq]/ml) was added to the cultures, and incubation continued for an additional 8 hours. The cells and media were then extracted with 4 M guanidinium hydrochloride, and the radioactivity incorporated into proteoglycans was separated from unincorporated precursor by size-exclusion chromatography on PD-10 columns (Pharmacia, Piscataway, NJ, U.S.A.).

[3 H]proline was used to label newly synthesized collagen and noncollagenous proteins (7). Cells were incubated for 24 hours with 5 μCi (185×10^3 Bq)/ml [3 H]proline (specific activity 55 Ci [$2,035 \times 10^9$ Bq]/mmol). At the end of the incubation period, newly synthesized proteins were recovered from the medium and cell layer and digested with highly purified collagenase ABC form III (Advanced Biofacturcs, Lynbrook, NY, U.S.A.) producing a soluble fraction representing collagen production. Collagenase-resistant material was digested with papain to release radioactivity incorporated into noncollagenous proteins. Because TGF- β_1 has been reported to increase the production of type-I collagen in other cell types (18,27), collagen phenotyping was performed to analyze the types of collagens produced in basal and TGF- β_1 -stimulated states. Collagen phenotyping was performed by a pre-

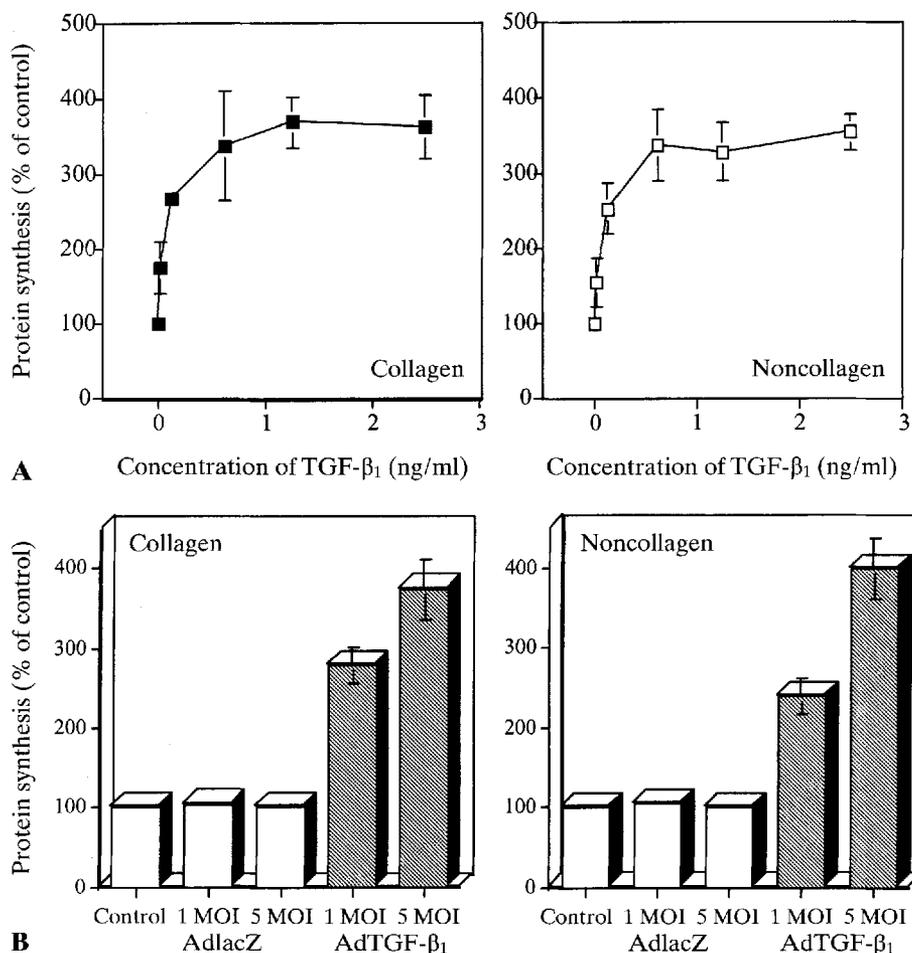


FIG. 3. Synthesis of collagen and noncollagenous proteins by chondrocytes in response to transforming growth factor β_1 (TGF- β_1) (A) exogenously added protein and (B) endogenously synthesized by transduced cells. **A:** Exogenous TGF- β_1 . Confluent cells were treated with different concentrations of purified, active TGF- β_1 for a total of 48 hours prior to determination of proteoglycan synthesis. The growth factor was replenished in the serum-free media after 24 hours. Data points are means of triplicate experiments ($n = 6$) \pm SD. **B:** Endogenous TGF- β_1 . Confluent chondrocytes were transduced with adenovirus containing the lacZ gene or cDNA encoding human TGF- β_1 (AdlacZ or AdTGF- β_1) at the multiplicities of infection (MOI) indicated and subsequently grown for 48 hours prior to determination of collagen and noncollagenous protein synthesis. Means of duplicate experiments ($n = 4$) are shown.

viously described method (7) following the separation of samples by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% gels. Briefly, extracts were treated with pepsin (100 μ g/ml) in 0.5 M acetic acid and digested for 4 hours. The digests were dialyzed against 0.05 M NH_4HCO_3 , dried, and analyzed by SDS-PAGE. Gels were treated with EN³HANCE (DuPont-NEN, Boston, MA, U.S.A.) prior to autoradiography. Densitometric analysis of autoradiograms was performed to determine the relative abundance of type-I and type-II collagen with the BIO-RAD GS-670 imaging densitometer (Bio-Rad Laboratories, Richmond, CA, U.S.A.). The positions of $\alpha 1(\text{I})$ and $\alpha 1(\text{II})$ chain migration on the gels were determined with purified type-I and type-II collagen run as standards on the same gels.

Matrix Metalloproteinase (MMP) Assays

Conditioned media were assayed for collagenase, gelatinase, and stromelysin with use of radiolabeled substrates (3). Casein (Sigma Chemical) and type-I bovine collagen (Vitrogen; Collagen Corporation, Palo Alto, CA, U.S.A.) were radiolabeled with tritiated acetic anhydride. Aliquots of [³H]collagen were heated to 60°C for 30 minutes to form gelatin. Assays were run in reaction buffer containing 50 mM Tris, 0.2 M NaCl, and 5 mM CaCl_2 , pH 7.4. Assay mixes contained 50 μ l radioactive substrate, 50 μ l con-

ditioned media, and 100 μ l reaction buffer. Aminophenylmercuric acetate (1 mM) was added to activate latent enzyme. Reactions were allowed to proceed for 4 hours at 37°C for caseinase (predominantly stromelysin) and gelatinase and 25°C for collagenase activity. The reactions were terminated by the addition of 250 μ l 10% trichloroacetic acid, and the radioactivities of the trichloroacetic acid soluble material were measured by liquid scintillation.

RESULTS

With use of a multiplicity of infection of 50, essentially all chondrocytes were positive for lacZ (data not shown). No toxicity was observed at any multiplicity of infection. Naive cells did not secrete detectable amounts of MMPs, and transduction of cells with adenovirus did not induce collagenase, gelatinase, or stromelysin activities (data not shown).

Cells infected with AdTGF- β_1 produced elevated amounts of TGF- β_1 protein as determined by ELISA (Fig. 1). Estimates of TGF- β_1 concentration by the mink lung cell bioassay provided values in close

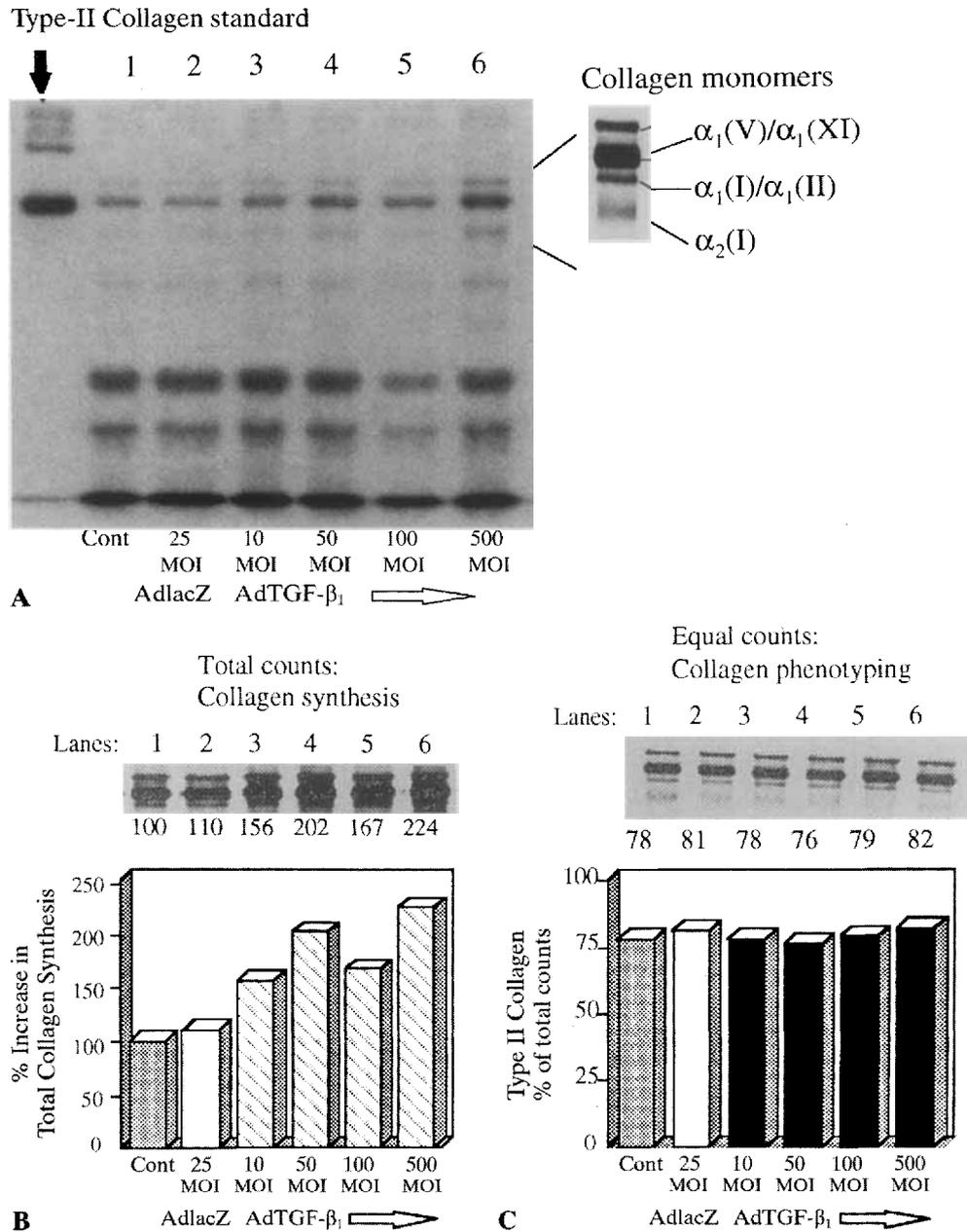


FIG. 4. Quantitative and qualitative effects of transduction with adenovirus containing cDNA encoding human transforming growth factor β_1 (AdTGF- β_1) on the collagen produced by chondrocytes. **A:** Coomassie blue-stained gel reveals the presence of a major collagen band migrating in the $\alpha_1(I)/\alpha_1(II)$ position. Autoradiography (inset) shows that in addition to type-II collagen chains, the newly synthesized collagen is composed of bands that co-migrate with $\alpha_1(V)/\alpha_1(XI)$ and $\alpha_2(I)$ chains. **B:** Densitometric scanning of protein bands on autoradiograms confirms an increase in collagen synthesis in response to TGF- β_1 produced by transduced chondrocytes. Values under each individual lane represent the amount of collagen synthesized, based on a control value of 100% (lane 1). **C:** Equal amounts of radioactive collagen (50,000 cpm/well) were loaded and separated on a 7.5% sodium dodecyl sulfate-polyacrylamide electrophoresis gel. Equal band intensities were obtained regardless of treatment. Densitometric scanning of the protein bands confirmed that 80% of the newly synthesized collagen was type II. The percentage of type-II collagen is given under each individual lane. In each panel, lanes 2-6 represent cells with increasing multiplicities of infection (MOI) of AdTGF- β_1 .

agreement with the ELISA data. Most of the TGF- β_1 in the conditioned media was latent (Fig. 1).

The addition of TGF- β_1 to cells maintained under serum-free conditions produced a dose-dependent, saturable response with a 225% increase in proteoglycan synthesis obtained with 2.5 ng/ml TGF- β_1 (Fig. 2). Cells transduced with AdTGF- β_1 also synthesized

proteoglycans at elevated rates, with 1 multiplicity of infection increasing production to 175% of control and 5 multiplicities of infection increasing production to 275% of control (Fig. 2B). These levels of stimulation are approximately equal to those obtained with the addition of purified protein at the concentrations achieved by gene transfer. The use of an adenoviral

control (AdlacZ) demonstrated that the effect was due to the presence of TGF- β_1 and not to the adenoviral transfection protocol. The stimulatory effect of the TGF- β_1 transgene was potentiated in the presence of 10% fetal bovine serum with the lowest concentration of virus, AdTGF- β_1 at 1 multiplicity of infection, increasing production 3-fold (Fig. 2B, serum).

TGF- β_1 also increased the synthesis of collagen and noncollagenous proteins in a dose-dependent, saturable fashion (Fig. 3A). Maximal increases in protein synthesis were obtained with 1.25 to 2.5 ng/ml TGF- β_1 , elevating the synthesis of collagen and noncollagenous protein to 362 and 353% of control, respectively. Adenovirally induced production of TGF- β_1 resulted in a dose-dependent increase in collagen (Fig. 3B) and noncollagenous protein synthesis. Thus, TGF- β_1 produced endogenously by chondrocytes as a result of gene transfer increased the synthesis of both proteoglycan and collagen to approximately the same degree as exogenously applied TGF- β_1 .

Separation of collagen molecules on the basis of molecular size was performed with SDS-PAGE (Fig. 4). The type-II collagen standard co-migrated with the major collagen band produced in untreated and infected primary chondrocytes. A band corresponding to the $\alpha 2(I)$ chain was barely visible on these Coomassie blue-stained gels, demonstrating that type-II collagen is the predominant collagen produced in these cells. The darker bands at the bottom of the gel in Fig. 4A are due to the addition of pepsin. Autoradiography was performed to establish the effect of TGF- β_1 treatment on collagen production. Total collagen synthesis was assessed, and an autoradiogram is shown (Fig. 4B). The increase in the density of bands generated after treatment of cells with AdTGF- β_1 confirms that adenovirally induced production of TGF- β_1 is capable of increasing collagen synthesis. The greater sensitivity of autoradiography compared with staining with Coomassie blue permitted detection of a small amount of $\alpha 2(I)$ collagen chains. Collagen phenotyping was performed on gels where equal counts were loaded (Fig. 4C). Band intensities for type-I and type-II collagens did not vary among treatment groups; densitometric analysis revealed an average of 80% type-II collagen produced in all groups on the basis of the assumption that the ratio of $\alpha 1(I):\alpha 2(I)$ chains is 2:1. This is the same percentage of type-II collagen as made by untreated, unpassaged chondrocytes (data not shown).

In view of the ability of TGF- β_1 to increase the synthesis of collagen by chondrocytes while maintaining the predominantly type-II collagen phenotype, we undertook a series of experiments to determine whether TGF- β_1 could rescue the collagen phenotype of cells that had been serially passaged. In these experiments, cells were exposed to TGF- β_1 from 48 hours to 5 days.

The representation of type-II collagen decreased from approximately 80% in unpassaged cells to 53% in cells at the second passage and 28% in cells at passage three (data not shown). The addition of as much as 2 ng/ml TGF- β_1 increased total collagen synthesis but failed to restore the representation of type-II collagen (data not shown). The TGF- β_3 isoform was also unable to elevate the proportion of type-II collagen synthesized by these cells (data not shown). Because TGF- β protein had no effect on the collagen phenotype of the passaged cells, experiments with transduced cells were not attempted.

DISCUSSION

These data confirm that articular chondrocytes are susceptible to transduction with adenoviral vectors, efficiently express a TGF- β_1 transgene delivered by such vectors, and respond to the secreted product by increasing matrix synthesis. The increment in matrix synthesis resulting from transgene expression was approximately equal to that achieved by adding a similar amount of purified human TGF- β_1 . Importantly, collagen synthesized in the presence of TGF- β_1 remained predominantly type II; it is well known that excessive production of type-I collagen leads to defective repair tissue that ultimately fails. Although TGF- β_1 was able to maintain the collagen phenotype of unpassaged cells, it could not rescue that of passaged cells, which produce predominantly type-I collagen. This observation is of relevance to the use of autologous chondrocyte transplants in repairing cartilaginous defects, where the cells undergo multiple cell divisions before reimplantation (6).

While this work was in progress, Arai et al. (2) reported complementary studies in which adenoviral transfer of a TGF- β_1 gene to a human chondrocytic cell line *in vitro* increased the abundance of aggrecan core protein mRNA while decreasing the abundance of stromelysin mRNA. They did not study collagen gene expression. As well as promoting the repair of cartilaginous lesions produced by trauma, the TGF- β_1 gene may be particularly useful in osteoarthritis, where the chondrocytes are hyper-responsive to TGF- β_1 (21). This is the opposite of their response to insulin-like growth factor-1 (IGF-1), which is depressed in osteoarthritis (22).

The intraarticular activity of TGF- β needs to be carefully controlled. Intraarticular injection of TGF- β protein, or delivery of AdTGF- β to the synovium, leads to inflammation, osteophyte formation, fibrosis, and cartilage erosions (1,12,33,34). In contrast, transfer of the TGF- β_1 gene to a limited number of cells within a cartilaginous lesion should allow for the local production of TGF- β_1 , the concentration of which will be high within the lesion but extremely low elsewhere. In this way, cartilage repair could be accelerated

without provoking additional adverse intraarticular disturbances.

Clearly, this approach can be used with genes other than TGF- β_1 . A variety of interesting growth factors, including certain members of the BMPs, fibroblast growth factor, IGF, platelet-derived growth factor, and hedgehog families of proteins, await evaluation (8). In addition, IL-1 suppresses cartilage repair by both inhibiting the synthesis and accelerating the breakdown of matrix. Baragi et al. (4) have shown that transfer of the IL-1 receptor antagonist (IL-1Ra) gene to chondrocytes protects cartilage from the catabolic effects of IL-1 *in vitro*. Delivery of the IL-1Ra gene to the synovial lining of joints ameliorates experimental models of rheumatoid arthritis and osteoarthritis (11) and is in a phase-I clinical trial for human rheumatoid arthritis (10).

The data presented in this paper encourage the further development of gene-based methods for preserving and repairing articular cartilage.

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