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Stimulating effect of diacerein on TGF-β1 and β2 expression in articular chondrocytes cultured with and without interleukin-1

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Summary

Objective: Diacetylrhein or diacerein has shown efficacy in the treatment of both major forms of osteoarthritis (OA), coxarthrosis as well as gonarthrosis, improving clinical symptoms of the disease (pain reduction and algo-functional index). Both in-vitro and animal models studies suggest that diacerein may have also disease-modifying effects. The drug exerts inhibitory effects on interleukin-1-induced expression of cartilage degrading enzymes. However, its mechanism of action is not completely understood. In view of the role that could play the transforming growth factor (TGF)-β system in the repair potentialities of OA cartilage, we studied the effect of diacerein on the expression of TGF-β isoforms 1, 2 and 3 and that of their receptor types I and II in cultured bovine chondrocytes.

Methods: Cultured bovine articular chondrocytes were treated with 10^{-5} M diacerein, 10 ng/ml IL-1β or the combination diacerein+interleukin (IL)-1, and the expression of both TGF-β isoforms 1, 2 and 3 and that of their receptors TβR-I and TβR-II was determined by Northern-blot and reverse transcriptase-polymerase chain reaction (RT-PCR). Cell transfections of cDNA constructs containing sequences of the 5′-upstream region of TGF-β1 promoter were also performed to determine their transcriptional activity in diacerein-treated cultures.

Results: The data indicated that diacerein enhances the expression of TGF-β1 and TGF-β2. This effect was also found in the presence of IL-1, albeit with smaller intensity. In contrast, the levels of TGF-β3 and receptors I and II remained unaffected or slighty modified by the compound. Treatment of cells transiently transfected with TGF-β1 promoter constructs suggested that the stimulating effect on TGF-β1 expression is mediated by the region −1038 to −1132 base pars.

Conclusion: The results suggest that diacerein effects on matrix synthesis and turn-over previously reported in cultured articular chondrocytes might be explained in part by the ability of the drug to enhance TGF-β1 and TGF-β2 expression in these cells. This mechanism of action may account for the potential disease-modifying properties of diacerein and might give clues as to how future anti-osteoarthritic drugs should be designed.

Key words: Articular chondrocytes, TGF-βs, TGF-β receptors, Diacerein, Interleukin-1.

Osteoarthritis (OA) is a degenerative disease that affects several joints and causes severe articular cartilage breakdown which may even lead to profound alterations of the underlying subchondral bone [1]. It is well documented that the process involves excessive production of metalloproteases (MMPs), such as collagenase and stromelysin, capable of degrading the cartilage matrix. Expression of these enzymes by the resident chondrocytes is induced by inflammatory cytokines, particularly interleukin-1 (IL-1) and, to some extent tumor necrosis factor-α (TNF-α) [2, 3]. Although the evolution of the destructive process remains irreversible so far, evidences have been reported that the cartilage may display anabolic signs in the early stages of OA, suggesting that the primary lesions can trigger the chondrocytes to proliferate and produce an excess of matrix macromolecules, including collagens and aggrecans [4–6]. This anabolic reaction has been interpreted as an attempt of repair, yet it is not clear whether the newly synthesized matrix displays similar characteristics to the normal pre-existing cartilage. Both in-vitro and in-vivo data support the hypothesis that the process is probably under the control of a growth factors network, of which the nature and functional mechanisms are practically unknown to date. However, among the various growth factors which have been found in the joint space and/or in the cartilage, transforming growth factor-β

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(TGF-β) appears as a good candidate to play a crucial role in the repair potentialities of cartilage, in view of its known stimulating effects on matrix synthesis which have been reported for various tissues and experimental models [7, 8]. Indeed, TGF-β is present in the cartilage matrix and is expressed by both chondrocytes and osteoblasts [9–11]. *In vitro*, it can enhance the expression of collagen type II and aggrecan by isolated chondrocytes as well as cartilage explants [12–14]. Furthermore, its injection in mice joints leads to increased proteoglycan synthesis in the articular cartilage [15, 16]. Taking into account that the erosion of cartilage is the prominent feature of OA, design of future strategies in the therapeutic treatment of the disease have so far essentially aimed at finding new ways to block the biologic activities of IL-1 in the joint tissues, for example by taking advantage of the IL-1 receptor antagonist (IL-1RA). However, an additional approach for control of OA would tend to stimulate the chondrocyte repair capacity, in order to compensate the cytokine-induced erosion of cartilage. Since TGF-β has been shown to antagonize the deleterious effects of IL-1, it is reasonable to think that stimulation of the TGF-β system in articular chondrocytes might be of great benefit to maintain their repair capacity in the OA process.

Therefore, drugs which could enhance the expression of growth factors implicated in the repair of articular cartilage may provide therapeutic approaches in the treatment of the disease. Diacerein (4,5-diacetoxy-9,10-dihydro-9,10-dioxo-2-anthracenecarboxylic acid), unlike common non-steroidal anti-inflammatory drugs, exerts its action through mechanisms which are not well understood but are probably not linked to the inhibition of prostaglandin synthesis [17, 18]. Diacerein has shown anti-OA effects in experimental animal models [19–21] and is currently used in the treatment of OA patients, with beneficial improvements of the clinical symtoms [22, 23]. The drug is marketed in France by Negma laboratories under the name ART® 50 and is presently evaluated as a disease-modifying OA drug (DMOAD) in a 3-year, randomized, double-blind, placebo-control clinical study of 500 patients with OA of the hip (ECHODIAH study)[24].

In vitro, diacerein has been shown to inhibit collagenase production by cultured rabbit articular chondrocytes and partially reverse IL-1-induced inhibition of proteoglycan synthesis [25, 26]. The drug, and its active metabolite rhein, have been reported to inhibit superoxide release from human neutrophils [27] and to reduce the phagocytic ability of mouse peritoneal macrophages [28]. All together, these data suggest that diacerein could interfere with the cytokines/growth factors network which plays a key role in the imbalance of cartilage metabolism during the OA process and may provide a rationale for the chondroprotective effects of the drug. To get further insights into the cellular mechanisms underlying this anti-OA action, we studied in-vitro effects of diacerein on the expression of TGF-β1, β2, β3 and their receptors I and II in cultures of bovine articular chondrocytes, in the presence and absence of IL-1.

Materials and methods

CULTURE AND TREATMENT OF ARTICULAR CHONDROCYTES

Chondrocytes were freshly isolated from calf cartilage as previously described [29] and used in primary cultures to minimize phenotype modulation. The cells were plated at $3–5 \times 10^4/cm^2$ in either 175-cm^2 flasks (RNA extraction) or six-well plates (9.6 cm^2) (TGF-β protein measurement). For transfection, the cultures were prepared at a seeding density of $15 \times 10^3/cm^2$ in 100-mm plates and used at approximately 75% of confluency. The cultures were incubated in Dulbecco's modified Eagle's medium (DMEM) (high glucose) containing 10% fetal calf serum (FCS) and antibiotics at 37°C in an atmosphere of 5% CO_2, 95% air, until the confluence was reached (except for transfection experiments). They were then incubated with diacerein (generally 10^{-5} M) provided by Negma Laboratories, (Toussus-le-Noble, Paris), and 10 ng/ml IL-1β (a generous gift from Dr Soichiro Sato, Shizuoka, Japan) for the indicated times. Since diacerein was dissolved in dimethylsulfoxide (DMSO), controls containing the same concentration of DMSO (1/1000 v/v) were included in all the experiments. These later were repeated at least twice and data from a representative set are shown.

RNA EXTRACTION

Total RNA was isolated by the acidified guanidium isothiocyanate method, using a commercial kit (RNAXel from Eurobio, France). The RNA concentrations were determined by measuring the OD_{260}. The OD_{260}/OD_{280} ratios were greater than 1.8. Integrity of RNA samples was controlled by running a 1% agarose gel electrophoresis in the presence of ethidium bromide. In case of contaminating genomic DNA, an additional precipitation with 6M LiCl was performed to obtain pure RNA samples.

NORTHERN HYBRIDIZATION

Ten micrograms of denatured RNA samples were run on a 1% agarose formaldehyde gel. The RNA was then transferred by capillary action to a nylon membrane (Pall Biodyne, Gelman Sciences) and immobilized by ultraviolet radiation (Bioblock UV Crosslinker, France). Two different probes were used to assess mRNA levels for TGF-β1 and β-actin: (a) a 336 base pair (bp) cDNA probe for TGF-β1 was generated by RT-PCR using the primers indicated below, (b) a 548 bp probe of β-actin generated by reverse transciptase-polymerase chain reaction (RT-PCR) with the specific primers listed below. The cDNA probes were radiolabeled using the Random Primers Labelling Kit (Gibco BRL, France) and [^{32}P]-dCTP (Amersham, France) as the radiolabel. Each probe was hybridized separately at 55°C and washed twice for 20 min at room temperature and once at 55°C with 0.1% SDS-containing 2 × SSC buffer. Signals were detected by contact film autoradiography using Kodak XAR5 film with intensifying screens. The relative optical density of the autoradiographic signals was normalized *versus* β-actin levels using two-dimensional laser scanning densitometry and ImageQuaNt software (Molecular Dynamics, France).

RT-PCR ANALYSIS

One-microgram samples of total RNA were reverse transcribed into cDNA in the presence of 100 pM antisense primer, 10 units of RNAsin (Promega), 10 mM dithiothreitol, 0.5 mM of each desoxynucleotide triphosphate (dNTPs) (Life Technologies), first strand buffer 5X and 60 units of Moloney murine leukemia virus-reverse transcriptase (Life Technologies). The reaction was performed at 42°C for 1 h. Amplification of generated cDNA was performed in an Omni E Hybaid thermocycler using the Life Technologies PCR kit, in the presence of both sense and antisense primers: **TGF-β1**, sense 5′-GCC CTG GAC ACC AAC TAT TGC-3′/antisense 5′-GCT GCA CTT GCA GGA GGG CAC-3′ [30]; **TGF-β2**, sense 5′-GCT TTG GAT GCG GCC TAT TGC-3′/antisense 5′-GCT GCA TTT GCA AGA CTT TAC-3′ [30]; **TGF-β3**, sense 5′-GCT TTG GAC ACC AAT TAC TGC-3′/antisense 5′-GCT ACA TTT ACA AGA CTT CAC-3′ [30]; **TβR-I**, sense 5′-ACG TTC GTG GTT CCG TGA G-3′/antisense 5′-TAA GTC TGC AAT ACA GCA AGT TCC ATT CTT-3′ [31]; **TβR-II**, sense 5′-CGC TTT GCT GAG GTC TAT AAG GCC-3′/antisense 5′-GAT ATT GGA GCT CTT GAG GTC CCT -3′ [32]; **β-Actin**, sense 5′-GTG GGG CGC CCC AGG CAC CA-3′/antisense 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′ [30]. Thirty-five cycles were done using the following conditions: 95°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. Then, an additional step at 72°C for 10 min was included. The number of cycles was selected in the exponential phase of the amplification curve previously established [33]. Transcripts were analyzed in 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The amplification reactions yielded expected transcript sizes (TGF-β1: 336 bp, TGF-β2: 336 bp, TGF-β3: 336 bp, TβR-I: 339 bp, TβR-II: 454 bp, β-actin: 548 bp). Identity of the PCR products was also confirmed by restriction endonuclease digestion and by southern-blot, using respective human probes which correspond, for TβR I and RII, to full-length cDNAs and for TGF-β1, TGF-β2, β-actin to fragments generated by RT-PCR. After photography of the gels with 665 Polaroid film, the intensity of corresponding bands was quantified by densitometric scanning performed with ImageQuaNt software (Molecular Dynamics) and normalized to β-actin mRNA levels.

MEASUREMENT OF SECRETED MATURE TGF-β BY GROWTH INHIBITION OF CCL-64 MINK LUNG CELLS

To measure the amount of active TGF-β in the media conditioned by control or diacerein-treated cells, confluent monolayer cells in six-well culture plates were incubated for 24 h in the 10% FCS-containing medium, with or without 10^{-5} M diacerein. The cells were washed three times with a serum-free medium supplemented with 200 µg/ml BSA (serum bovine albumin) for 5, 30 and 60 min, and incubated in 1 ml of the serum-free medium for an additional 6 h period. The conditioned medium was then collected in siliconized microcentrifuge tubes, centrifuged and kept at −70°C until use. Before the assay, 0.5 ml of cell culture supernate was acidified by adding 0.1 ml of 1 N HCl, incubated for 10 min at room temperature and neutralized with 0.1 ml of 1.2 N NaOH/0.5 M HEPES. CCL-64 cell cultures in proliferative state were prepared as described previously [34], by seeding 2×10^4 cells/well in 24-well plates and grown in 10% FCS-medium. Twenty-four hours later, they were washed with serum-free medium and incubated for 24 h with 0.5 ml of chondrocyte conditioned media or increasing concentrations of exogenous TGF-β1 to establish a reference curve of growth inhibition. The cultures were pulsed with 0.5 µCi/ml of [^{3}H]thymidine (40–60 Ci/mmole, NEN., France) over the last 4-hour period of incubation. Cells were fixed with cold 5% (v/v) trichloracetic acid, rinsed with the same solution

and dissolved in 0.1 N NaOH before counting in scintillation liquid.

To check the specificity of the assay, CCL-64 cells were prepared as described above. Twenty-four hours after seeding, they were preincubated for 30 min at 37°C with increasing concentrations of TGF-β_1, β_2, and β_3 antibodies (R & D Systems). Thereafter, exogenous TGF-β_1, β_2, and β_3 (0.5 µg/ml) were added for 24 h and the cells were pulsed with tritiated thymidine as described for conditioned media.

CELL TRANSFECTION AND LUCIFERASE ACTIVITY ASSAY

For transient transfections, cells were plated in 100-mm Petri dishes and grown to 70–80% of confluence. Cells were then cotransfected by the calcium phosphate coprecipitation method [35], with 9 µg of the appropriate plasmids and 3 µg of pSV40-β Gal (Promega), a β galactosidase expression vector used as an internal control to normalize for transfection efficiency. After 24 h, the medium was changed to medium containing DMSO (1/1000) in the absence or presence of 10^{-5} M diacerein. Cells were harvested 48 h after DNA addition and extracts were assayed for luciferase activity. Briefly, the dishes were washed twice with PBS, and the cells were lysed with 300 µl of lysis buffer (0.45 mM Tris-HCl, pH 7.5). The lysates were submitted to three cycles of freeze–thaw. After centrifugation, luminescence of a 50-µl aliquot of lysate from each dish was measured in a luminometer (Berthold Lumat 9501) for 20 s after the addition of luciferin (Luciferase Assay System, Promega). To normalize the luciferase activity, protein concentration and β galactosidase activity were determined. Protein concentration of 4 µl cell lysates from each dish was measured by the Bradford's method [36]. Cell lysates were assayed for β galactosidase activity using resofurin β-D galactopyranoside as substrates and the OD_{572} measured.

CONSTRUCTION OF PLASMIDS

The TGF-β1 promoter constructs were generated using plasmid phTG2 [37] kindly provided by Dr S. J. Kim (Laboratory of Chemoprevention, NIH/NCI; Bethesda). phTG2 was digested by various restriction endonucleases and the DNA fragments were then cloned into a promoterless luciferase reporter plasmid, basic pGL2 (Promega). Constructs containing sequences from −1132 to +11, −732 to +11, were obtained by digestion of phTG2 respectively with HindIII and Xba I. After filling in the ends with Klenow fragments, these DNA sequences were digested with Kpn1 and cloned into a SmaI-Kpn1 site of the plasmid basic pGL2. The −454/+11 construct was prepared as a HincII-Kpn1 restriction fragment from phTG2 and was cloned into a SmaI-Kpn1 site of the plasmid basic pGL2.

Results

EFFECT OF DIACEREIN ON mRNA EXPRESSION OF TGF-β1, β2 AND β3

We first determined the effect of diacerein on the steady-state level of TGF-β1 mRNA, using a 24 h treatment period and a concentration of 10^{-5} M, which has been previously shown to be within the range of pharmacological blood levels measured in patient treatment [38] and also capable of acting in similar experimental conditions on the expression of other genes, such as collagenase, by cultured chondrocytes [25]. Interleukin-1 was added alone or in combination with diacerein in order to estimate the influence of this cytokine which is known to play a crucial role on chondrocytes in the OA process.

As illustrated in Fig. 1, diacerein induced a dramatic increase in TGF-β1 mRNA level. IL-1β did not significantly affect the message for TGF-β1. Interestingly, in the presence of IL-1, diacerein was still capable of enhancing TGF-β1 expression, but to a lesser extent. Additional experiments were then carried out to evaluate the effects of diacerein on the message levels for the other isoforms of TGF-β, TGF-β2 and TGF-β3. In that case, we preferentially used RT-PCR instead of Northern-blot technique as better detection was observed with the former method, probably because TGF-β2 and TGF-β3 were much less expressed by the chondrocytes than was TGF-β1. As represented in Fig. 2, TGF-β2 mRNA level was also increased by diacerein. However, upon stimulation with IL-1β, the effect was no more visible, the intensity of the band being rather similar to that of IL-1 alone. Under these experimental conditions, the level of TGF-β3 message remained unchanged.

EFFECT OF DIACEREIN ON THE RELEASE OF MATURE TGF-β

To determine whether the increase of mRNA levels observed for TGF-β1 and TGF-β2 was accompanied by an augmentation of the protein synthesis, we assayed the production of total mature TGF-β released into the culture medium under similar conditions. After activation of the supernatants by acidification, the samples were

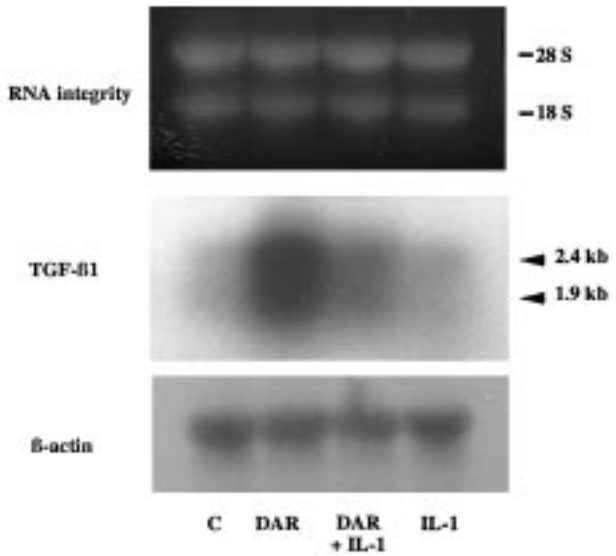

FIG. 1. Effect of diacerein and IL-1 on expression of TGF-β1. Bovine articular chondrocytes were plated at 6×10^6 cells per 175-cm^2 flasks in DMEM containing 10% FCS. The media were changed every two days until the cultures reached confluency. At that time, they were treated for 24 h with 10^{-5} M of diacerein (DAR) and 10 ng/ml of IL-1 alone or in combination. Thereafter, total RNA was extracted and 10 µg were run on 1% agarose gel electrophoresis and transferred to nylon membrane. The filter was hybridized successively with TGF-β1 and β-actin probes as described under 'Materials and methods'.

evaluated on the proliferation rate of CCL-64 epithelial cells in their exponential growth phase. As can be seen on Fig. 3, incubation of chondrocytes with 10^{-5} M diacerein for 24 h resulted in a six-fold increase of TGF-β production, demonstrating that transcriptional effect of the drug is associated with a corresponding translational activity.

EFFECT OF DIACEREIN ON CELLULAR EXPRESSION DIRECTED BY THE 5′ FLANKING REGION OF THE TGF-β1 GENE

In view of the preceding results which suggested that diacerein was able to stimulate the transcriptional activity of the TGF-β1 gene promoter, we carried out transient transfections of the chondrocytes with a series of deleted fragments of the 5′-promoter region of the human TGF-β1 gene fused to the luciferase reporter gene in order to delineate the *cis*-sequences that could mediate the effect of the compound. The constructs were transfected into the cell cultures which were then treated for 24 h with 10^{-5} M diacerehin. The expression of the luciferase activity was measured for each plasmid. As shown in Fig. 4, the largest construct used (−1132 to +11) induced a 13-fold increase of activity compared to the control. The

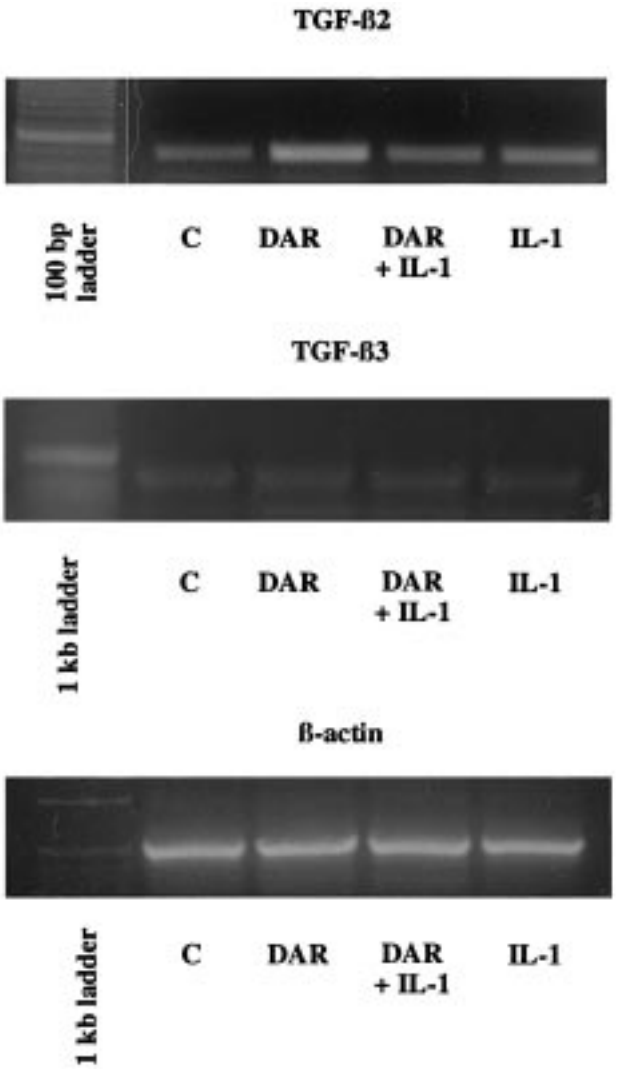

FIG. 2. Effect of diacerein and IL-1 on expression of TGF-β2 and β3. Articular chondrocytes were cultured as in Fig. 1, and 1 µg of total RNA was reverse trancribed into cDNA by using specific antisens primer for TGF-β2, β3 and β-actin. Thirty-five PCR cycles were performed under conditions described in 'Materials and methods' as deduced from preliminary amplification curves. Products were analysed in 2% agarose gel electrophoresis in the presence of ethidium bromide and photographied under UV light.

other sequences corresponding to the more downstream region (−1038 to +11) did not produce significant change in the luciferase expression. To determine if the metabolite of diacerein, rhein, was also capable of stimulating the transcriptional activity of the TGF-β1 promoter, two of the constructs (−1132 and −454 bp) were used and the transfected cultures exposed directly to rhein. A three-fold increase of activity was observed with rhein on the largest construct, indicating that rhein exerts the same effect but to a lesser extent (Fig. 4, insert). These data suggest that the DNA

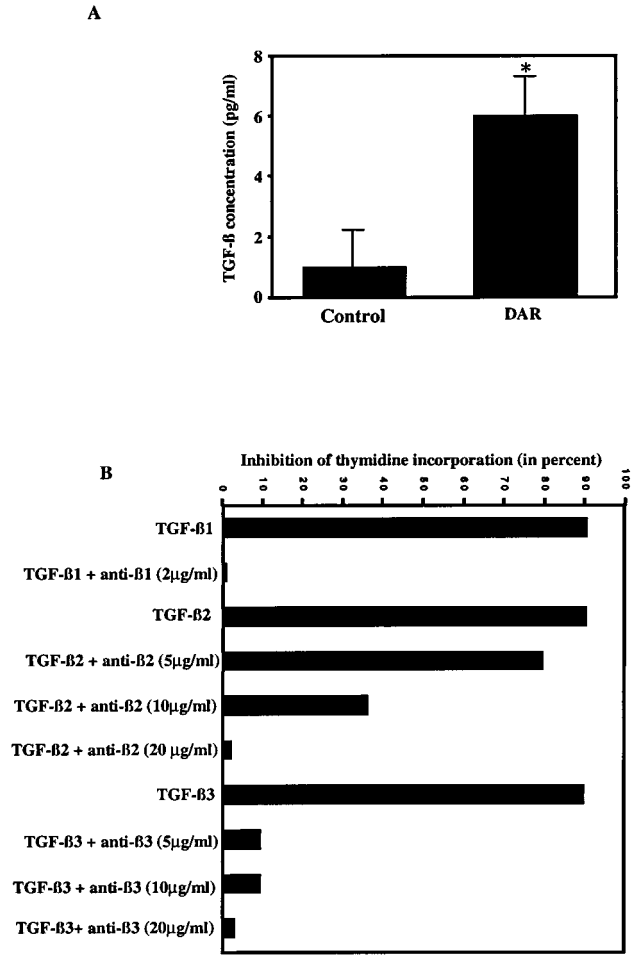

FIG. 3. Effect of diacerein on expression of TGF-β protein. The measurement of active TGF-β proteins was performed after a 24-h treatment with 10^{-5} M diacerein, three washes with serum free medium supplemented with BSA and a final 6 h-incubation with 1 ml of the same medium (see 'Materials and methods'). The remaining supernatant was activated by acidic treatment and total TGF-β activity was determined by the CCL64 assay. A dose-response reference curve has been established with exogenous TGF-β_1. Values are means ± S.E.M. (N=3) and significance of the difference between treated and control cultures was established by Student's t-test (*$P \leq 0.05$). A: Results were obtained as percentage of tritiated thymidine incorporation inhibition and expressed as amount of produced TGF-β. B: Parallel cultures were prepared and preincubated for 30 min with TGF-β1 (anti-β1), TGF-β2 (anti-β2) and TGF-β3 (anti-β3) respective antibodies at increasing concentrations. Thereafter, TGF-β1, β2 and β3 were added at final concentration of 0.5 ng/ml and the proliferation was measured by tritiated thymidine incorporation as above.

sequences responsible for the diacerein stimulating effect on TGF-β1 gene expression are located between −1132 and −1038 bp. Further analysis is presently undertaken to more precisely delineate this regulatory region.

EFFECT OF DIACEREIN ON THE EXPRESSION OF TGF-β RECEPTORS

As the biological activity of TGF-β is mediated through its binding to cell membrane receptors, we explored the effect of diacerein on the expression of TGF-β receptors by measuring the corresponding mRNA levels. Figure 5 shows the results of a typical RT-PCR analysis where the data were obtained from the exponential phase of amplification and normalized to the levels of β-actin determined in parallel. The message for TβR-I was increased by 10^{-4} M diacerein at 12 h. IL-1 has a tendency to reduce the level of TβR-I mRNA, an effect which seems to be reinforced in the presence of diacerein. At 24 h, no significant change was observed for TβR-I message except that IL-1 enhanced the values when added alone to the cultures. TβR-II mRNA levels remained unaffected by the treatments at 12 h. In contrast, all the agents exerted an inhibitory effect at 48 h. In particular, IL-1 was shown to constantly decrease the expression level of TβR-II at this time of exposure.

Discussion

The potential role of TGF-βs and their related peptides BMP (bone morphogenetic peptides) in the control of both cartilage homeostasis and repair has been extensively explored over the last few years. Several in-vitro studies have demonstrated that this growth factor can counteract the effects of interleukin-1 on the catabolism as well as on the synthesis of chondrocyte matrix components (reviewed in [8]). Furthermore, it has been shown that intra-articular injections of TGF-β into mice joints induced an increase of cartilage proteoglycan synthesis and even promoted formation of osteophytes if the dose and number of injections are too high [15, 16]. The recent finding that the expression of TGF-β receptor II is dramatically reduced to barely detectable levels in the fibrillated cartilage of the rabbit OA model [39] and that transgenic mice expressing a truncated, kinase-defective, TGF-β type II receptor develop a degenerative joint disease resembling human OA [40] places greater emphasis on the role of this factor in this disease. From these data, it seems probable that the OA process is accompanied by a progressive reduction of chondrocyte responsiveness to TGF-β and therefore an incapacity to counter the IL-1-induced degradation. In this context, any agent that could stimulate the expression of TGF-βs or the signaling pathway of these factors in cartilage may have some interest for OA treatment and provides a valuable contribution to the

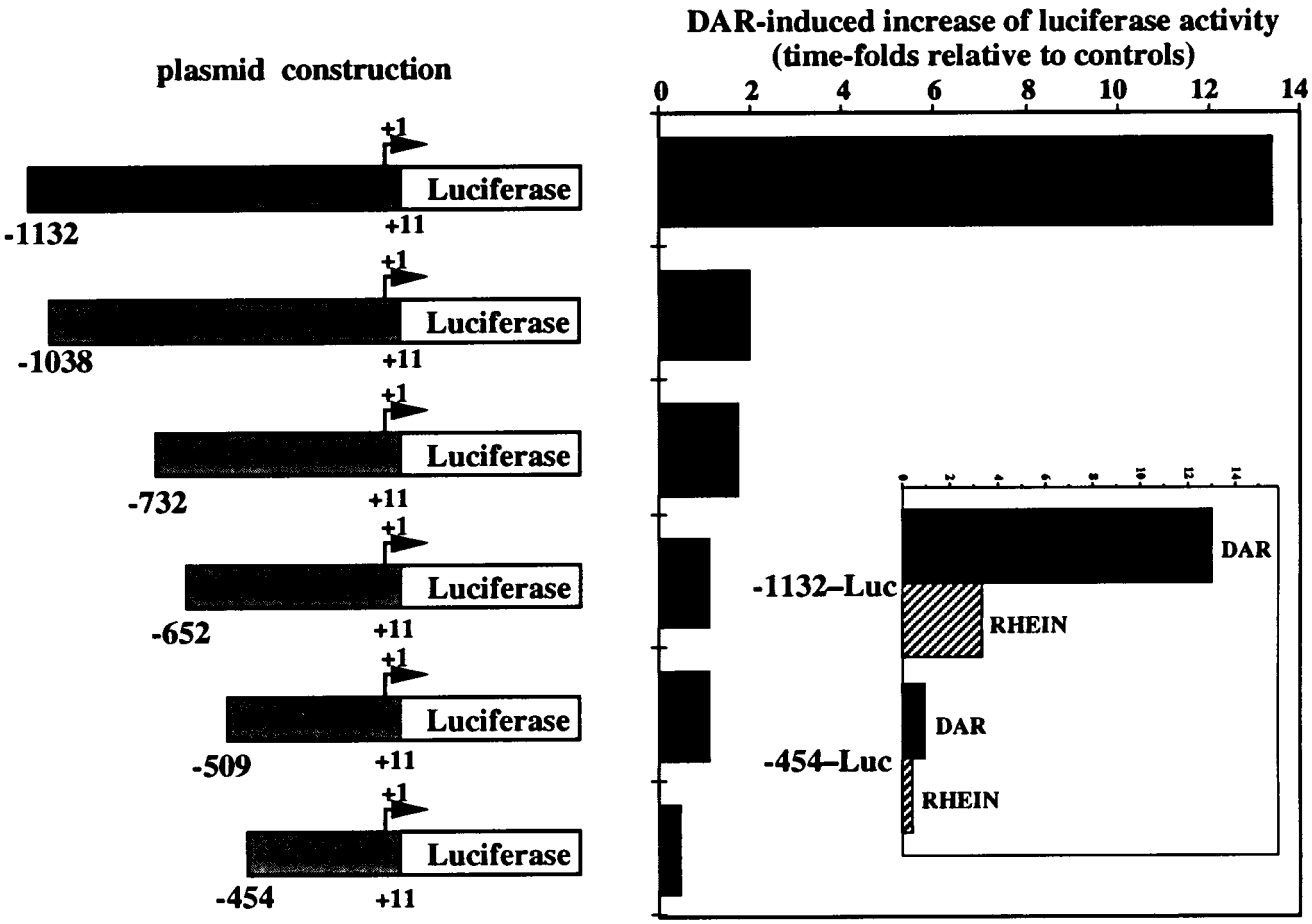

FIG. 4. Effect of diacerein on cellular expression directed by the 5′ flanking region of the TGF-β1 gene. Bovine chondrocytes were plated at a density of 1.2×10^6 in 55 cm^2 Petri dishes and grown in DMEM containing 10% FCS to 70–80% of confluency. Cells were then cotransfected with 9 μg of the different TGF-β1 promoter constructs and 3 μg of pSV40-β Gal. 24 h after the transfection, the cultures were washed and treated or not with diacerein 10^{-5} M (and rhein 10^{-5} M, see insert). 48 h after DNA addition, the cells were harvested and luciferase activity was determined as relative light units (RLU) normalized to β galactosidase activity and protein concentration. Results are expressed as diacerein/rhein-induced increase of luciferase activity. Values are means ± S.E.M. (N=3).

development of new therapeutic strategies in this field. In the present study, we have demonstrated that diacerein and its active metabolite rhein can, at therapeutic concentrations, increase the expression of TGF-β1 and TGF-β2. This effect was consistent throughout the study but was shown for concentration thresholds and exposure times slightly variable depending on the chondrocyte strains. This variation is probably related to the fact that, depending on the age of animals from which the cells are obtained, the responsiveness of chondrocytes may be different. Moreover, the rate of diacerein metabolization into rhein, the active derivative, is likely to depend also on the cell strain and therefore may vary between experiments. However, the drug concentrations used here and shown to be efficient on TGF-β1 and β2 expression are well within the therapeutic range of OA patients treated with diacerein [38].

Interestingly, we demonstrate also that diacerein was still capable to exert its enhancing effect on TGF-β1 gene expression, although to a lesser extent, in the presence of IL-1, an experimental condition which mimics in some way the situation encountered by chondrocytes in OA cartilage. It must be remembered that we previously showed an inhibitory effect of diacerein on the IL-1-induced collagenase synthesis by chondrocytes [25, 26]. Thus, the present finding is consistent with a possible mechanism linking the action exerted by the drug on both cytokine specific effects. By stimulating the expression of TGF-β1 and -β2, diacerein can first trigger the feedback loop of auto-induction of TGF-β1 [41], amplifying its sphere of activity, and secondly may counteract the IL-1-induced degradative process [8].

It is of note that in our study, the stimulating effect of diacerein was generally greater on the

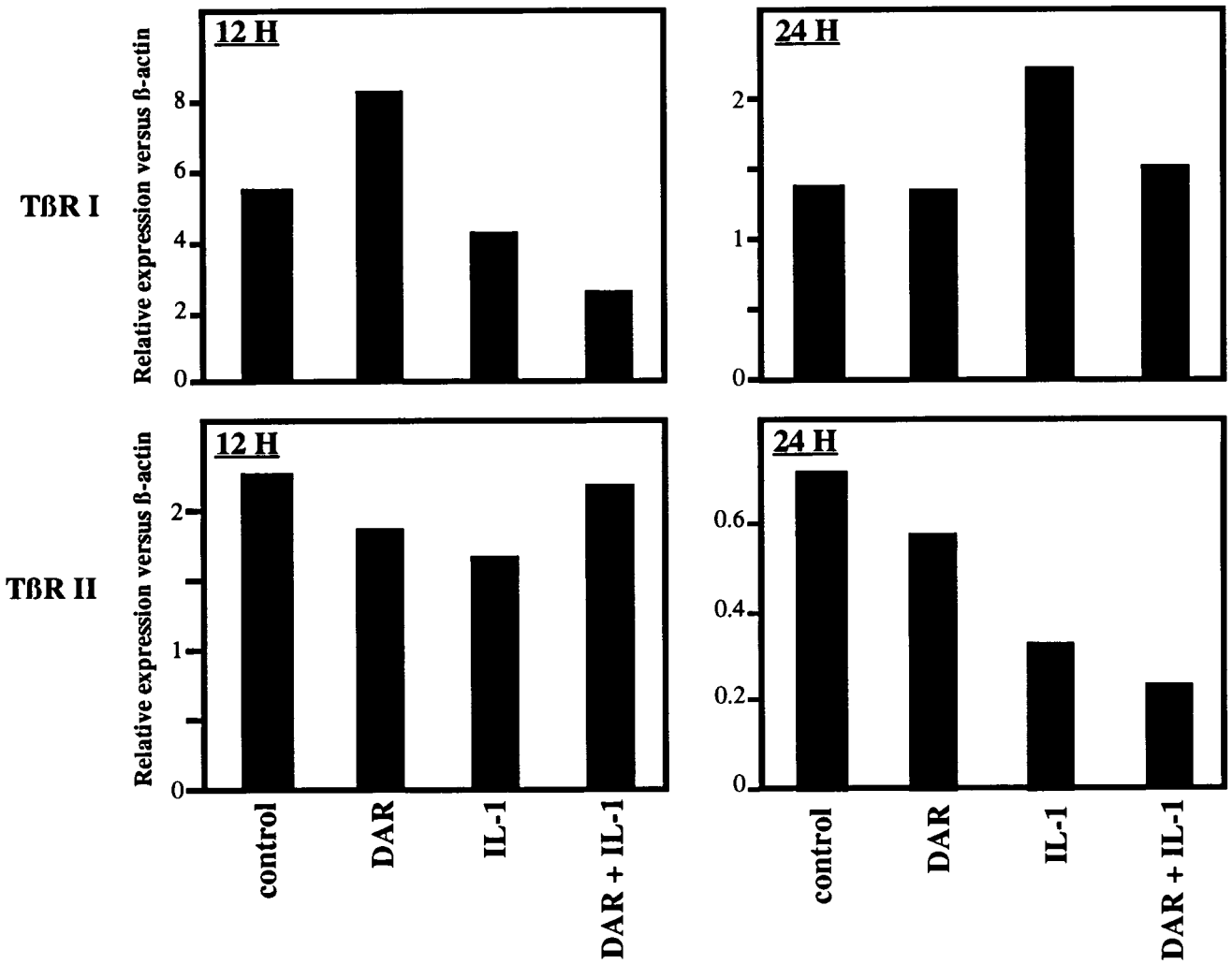

FIG. 5. Effect of diacerein and IL-1 on the expression of TGF-β receptors I and II. Bovine articular chondrocytes were cultured as described above in 175 cm^2 flasks treated at confluency with 10^{-5} M diacerein and 10 ng/ml of IL-1 alone or in combination. Total RNA was extracted after 12 and 24 h of incubation. 1 μg were used for reverse transcription of both TGF-β receptors type I and type II and β-actin. PCR reactions were carried out for increasing numbers of cycles (25, 30 and 35) in order to check the linearity of amplification. Products of the amplifications were run on 2% agarose gel electrophoresis and signals were scanned for further analysis with ImageQuaNT software (Molecular Dynamics). Results are expressed as histograms of TβR I or TβR II relative expression *versus* that of β-actin.

expression of TGF-β1 than of TGF-β2. This finding is most important, as several evidences have been accumulated that expression of TGF-β1 is differently regulated than the two other isoforms [42, 43]. Characterization of the promoters of human TGF-β1, 2 and 3 has revealed significant differences in the regulatory features of these three promoters [37]. The TGF-β1 promoter contains no TATA box but instead a cluster of Sp1 sites just upstream from the transcriptional start sites. The three Ap-1 sites, which mediate autoinduction of TGF-β1 [41], seem to be critically important in the regulation of TGF-β1 expression in a variety of physiological and pathological conditions and could explain perhaps that TGF-β1 expression appears to be more sensitive to cytokines/growth factors control than those of TGF-β2 and β3. Since only the TGF-β1 gene has been found to contain functionally active AP-1 binding sites, it has been suggested that many of the increases in TGF-β1 transcription observed in transformed cells, in wound healing, and in chronic inflammatory diseases might be mediated through activation of the AP-1 sites by Jun and Fos, either directly or indirectly.

Transfection experiments presented here suggest that the stimulating effect of diacerein on TGF-β1 expression could be mediated through positive regulatory sequences located between −1132 and −1038 bp of the 5′-upstream region of the promoter. This region is therefore different from the zone −453/−333 bp previously identified

as the zone responsible for auto-induction and which contains the major consensus transcription factor binding site, AP-1 [37]. However, the −1132/−1038 region shown here to be implicated in the effect of diacerein corresponds precisely to the sequence which contains an enhancer-like zone capable of compensating the more downstream negative regulatory region −731/−443 [37]. This region, which has not been yet investigated, does not contain distinct recognizable transcriptional factor binding sites but GC-rich sequences are present, suggesting that they could be putative binding sites for zinc-finger proteins. We are currently attempting to further delineate this sequence and determine which transcription factors might be implicated at this level.

Under the present experimental conditions, no clear and significant effects of diacerein on the expression of both TGF-β receptors I and II were observed, and there is a doubt that the slight variations we found could have some influence on the factor binding potentialities of the chondrocyte and the subsequent signaling pathways. In contrast, we found a reproducible negative effect of IL-1 on the expression of the TGF-β receptor II which is known as the crucial part of the cell binding system. This data is consistent with the fact that chondrocytes may become less sensitive to TGF-β in the OA process where excess of IL-1 is known to be a major characteristic of the affected joints.

To date, very few drugs have proven capable of stimulating TGF-β expression. Taken into the context of the recent findings supporting the role of TGF-β in cartilage repair potentialities [39, 40], this study may explain some of the mechanisms through which diacerein exerts a therapeutic effect in OA. Together with its already reported inhibitory effect on IL-1-induced collagenase expression, its capability to stimulate TGF-β expression points to the potential interest of this drug to the repair of cartilage.

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