

Anabolic actions of IGF-I and TGF- β 1 on Interleukin-1 β -treated human articular chondrocytes: Evaluation in two and three dimensional cultures

Claudia Seifarth, Constanze Csaki and Mehdi Shakibaei

Musculoskeletal Research Group, Institute of Anatomy, Ludwig-Maximilian-University Munich, Munich, Germany

Summary. Pro-inflammatory cytokines, such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) play a key role in the pathogenesis of Osteoarthritis (OA). The aim of this study was to investigate the potential anti-inflammatory properties of the growth factors IGF-I and/or TGF- β 1 on IL-1 β signalling pathways and their effect on the chondrogenic potential of dedifferentiated human articular chondrocytes *in vitro*.

Serum-starved human articular chondrocytes were treated with IL-1 β to induce dedifferentiation and further treated with IGF-I and/or TGF- β 1 at various concentrations. The effects of growth factors were evaluated both in monolayer and high-density cultures.

Incubation with the cytokine IL-1 β resulted in rapid dedifferentiation of the cells; they lost their chondrocyte-like phenotype while down-regulating the expression of collagen type II, integrin, extracellular regulated kinase (Erk 1/2) and the chondrogenic transcription factor Sox9. Co-treatment with IGF-I and/or TGF- β 1 stimulated the cells to redifferentiate, increasing the expression of the above-mentioned cartilage-specific proteins. These events correlated with down-regulation of cyclooxygenase-2 (COX-2) and matrix metalloproteinase-13 (MMP-13). Furthermore, in high-density cultures, we observed evidence for new cartilage formation after co-treatment with these growth factors. We further detected that all examined proteins were more strongly expressed during combination treatment.

These results indicate that IGF-I and TGF- β 1 exert additive anabolic effects on chondrocytes and may stabilize the chondrogenic potential. The additive action of these growth factors on chondrocytes may find

practical applications in the fields of OA and cartilage tissue engineering.

Key words: Human cartilage, Chondrocyte, Osteoarthritis, ACI, IL-1 β , TGF- β 1, IGF-I, Chondrogenesis

Introduction

In modern societies, we observe an increasing number of people suffering from degenerative joint diseases, often leading to major impediments in everyday life for these patients and high costs for the welfare systems. Degenerative influences trigger pathologic changes to chondrocyte function and phenotype during Osteoarthritis (OA). Pro-inflammatory cytokines released from activated synoviocytes and articular cartilage cells promote degradation of extracellular matrix compounds (Feldmann et al., 1996; Fernandes et al., 2002). Many investigators have demonstrated that cytokines, like IL-1 β and TNF- α , are produced in high amounts during OA in the cartilage and in turn stimulate the synthesis of other pro-inflammatory catabolic factors and mediators of degradation and inflammation, such as MMPs (Ryu et al., 1984; Robbins et al., 2000; Fernandes et al., 2002) and COX-2 (Largo et al., 2003). However, IL-1 β may also contribute to the depletion of cartilage matrix by decreasing the synthesis of cartilage-specific proteoglycans and collagen type II (Studer et al., 1999; Goldring, 2000; Robbins et al., 2000). Apoptosis is another contributing factor to the loss of articular cartilage in OA and it is known that apoptosis increases the cell loss observed in aging and OA cartilage (Goldring, 2000).

Recently, the Autologous Chondrocyte Implantation (ACI) has been proposed as the gold standard for the repair of articular cartilage lesions (Brittberg et al.,

1994). However, although results obtained until now are quite encouraging (Peterson et al., 2002), the main limitation for successful ACI is often the poor integration of the newly formed cartilage tissue. This is mainly due to the expansion phase in monolayer culture where chondrocytes gradually lose their specific phenotype, dedifferentiate into fibroblast-like cells and thus lose their chondrogenic potential (von der Mark et al., 1977; Quarto et al., 1990; Shakibaei, 1995; Shakibaei et al., 1997). These dedifferentiating processes lead to chondrocytes that are incapable of initiating cartilage defect repair, resulting in fibrous cartilage tissue. However, the expansion phase is necessary, since primary chondrocyte yields from biopsies are low and do not suffice to refill cartilage defects immediately. For this reason, new and improved *in vitro* strategies need to be investigated.

It has been observed that after monolayer expansion up to the fourth passage, chondrocytes are able to redifferentiate spontaneously in three dimensional surroundings, such as in alginate or high-density culture (Schulze et al., 2000; Schulze-Tanzil et al., 2002, 2004). Growth factors are known to exert anabolic effects on chondrocytes *in vivo* and *in vitro* by influencing chondrocyte proliferation, differentiation, growth and survival (Mandl et al., 2004). Especially, members of the TGF-superfamily, such as Transforming growth factor- β 1 (TGF- β 1), have more and more come into focus as potential candidates for enhancing cartilage repair. TGF- β 1 is known to be expressed in normal articular cartilage, where it is able to augment cell proliferation. Furthermore, it influences and enhances the production of cartilage matrix-specific proteins (Denker et al., 1995). Insulin-like growth factor-I (IGF-I) has been shown to augment the synthesis of cartilage-specific matrix proteins *in vivo* and *in vitro* (Yaeger et al., 1997; Mi et al., 2000; Shakibaei et al., 2006). Furthermore, IGF-I, as well as TGF- β 1, strongly promote chondrogenesis in progenitor cells such as mesenchymal stem cells *in vitro* (Fukumoto et al., 2003; Longobardi et al., 2006).

Because of the well established and potent anabolic effects of these growth factors in cartilage and because only limited investigations have been done in cartilage metabolism and combined growth factor therapy, in this study we wanted to examine the influence of IGF-I and TGF- β 1 on restoring the chondrogenic potential of dedifferentiated chondrocytes from monolayer culture. Furthermore, this study suggests the usefulness of cytokine inhibition or inhibition of cytokine downstream factors by growth factors during the treatment of OA, thereby improving chondrocyte cultivation *in vitro* and thus the success of ACI.

Materials and methods

Antibodies

Antibodies against collagen type II, cartilage-specific proteoglycan and β_1 -integrin, as well as

secondary antibodies, were purchased from Chemicon International (Temecula, CA, USA). Antibodies to active caspase-3 and MMP-13 were from R&D Systems, Inc., (Wiesbaden, Germany). Sox9 antibody was purchased from Acris Antibodies GmbH (Hiddenhausen, Germany), Phospho-p42/p44 Erk1/2 from R&D, COX-2 antibody from Cayman (Michigan, USA) and anti- β -actin antibody from Sigma (Munich, Germany).

Growth medium and cytokines

The applied growth medium was obtained from Seromed (Munich, Germany). It consists of Ham's F-12 and Dulbecco's modified eagles medium containing 10% fetal calf serum (FCS), 25 μ g/ml ascorbic acid, 50 IU/ml streptomycin and penicillin, 2.5 μ g/ml amphotericin B, 1% essential amino acids and 1% L-glutamine. Alginate and trypsin/EDTA were purchased from Sigma (Munich, Germany), IL-1 β from Strathman Biotech GmbH (Hannover, Germany) and IGF-I and TGF- β 1 from Pepro-Tech (London, UK).

Chondrocyte isolation and culture conditions

Primary chondrocytes were isolated from articular cartilage obtained during joint replacement surgery of femoral head fractures as described earlier (Shakibaei et al., 1999). Cartilage samples were derived from three middle-aged patients and used for the investigations. After isolation, the cells were re-suspended in growth medium and cultured in a three dimensional alginate system as previously described (Shakibaei and De Souza, 1997). Briefly, one million cells were re-suspended in 1 ml of alginate. This solution was dripped into a calcium-chlorid solution where it polymerised to beads. After washing with buffer, these beads were cultured in whole cell culture medium containing 10% FCS. After a few days of culturing, the chondrocytes migrated from the alginate and adhered to petri dishes in monolayers (Schulze-Tanzil et al., 2004). Studies from our laboratory have shown that alginate functions as a selective filter station separating vital from non-vital chondrocytes and fibroblasts (Schulze-Tanzil et al., 2002, 2004). These cells were used for our experimental design. For all the experiments described these emigrated chondrocytes were passaged only twice to circumvent dedifferentiation. Gradually the culture medium was converted into serum-starved medium containing only 0.5% FCS.

Experimental design

The experiments performed in this study were performed on chondrocytes isolated from three individual donors (n=3) and specifically designed to mimic cellular events that occur in the clinical condition of OA: to induce chondrocyte dedifferentiation processes in monolayer cultures we adopted a model which stimulates chondrocytes with the pro-inflammatory cytokine IL-1 β (10 ng/ml). During

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monolayer expansion chondrocytes were cultured in whole cell culture medium containing 10% FCS. Before starting the experiments described here, chondrocytes were washed three times with serum-starved medium (containing only 0.5% FCS) and further incubated for 30 minutes with serum-starved medium before starting the IL-1 β treatment. Serum-starved chondrocytes were stimulated with (10 ng/ml) IL-1 β alone for 24 hours before being co-treated with 10 ng/ml IL-1 β and IGF-I (100 ng/ml) or TGF- β 1 (100 ng/ml) or the combination of both growth factors at various concentrations (10, 100 ng/ml) for an additional 24 or 48 hours. Growth factor concentrations used in the presented experiments were determined in previous studies from our own laboratory through dose dependent experiments on human articular chondrocytes. Furthermore, in a second approach, monolayer cultured chondrocytes, treated as presented above, were transferred to high-density cultures and cultured under identical conditions with serum-starved medium, in order to examine the effects of IGF-I and/or TGF- β 1 on chondrocyte redifferentiation potential in a three dimensional environment. Three dimensional high-density culture was performed as previously described by Shakibaei et al. (1993). Briefly, one million cells were pipetted onto a nitrocellulose filter resting on a steel net bridge. Cell culture medium reached the filter medium interface and cells were nurtured through diffusion. After one day in culture cells aggregated and formed a pellet on the filter (Shakibaei et al., 1993, 1995; Shakibaei and Mohamed-Ali, 1994). After seven days of culture, cultures were prepared for further investigations.

Western blot analysis

Western blot analysis were performed three times for each donor as previously described (Shakibaei et al., 2006). After adjusting the equal amounts of total proteins they were separated by SDS-PAGE under reducing conditions and transferred onto nitrocellulose membranes. Specific binding was quantified by densitometry using "quantity one" (Bio-Rad Laboratories Inc. CA, USA).

Transmission electron microscopy

Cells were fixed in Karnovsky-fixative for 30 minutes and post-fixed in a 1% OsO₄ solution. Chondrocytes were dehydrated in an ascending alcohol series before they were embedded in Epon, and cut on a Reichert Ultracut. 2% uranyl acetate/lead citrate was used for contrasting these ultrathin sections, as previously described (Shakibaei et al., 1995; Shakibaei, 1998).

Statistical analysis

Results obtained in this study are expressed as the means \pm S.D. of a representative experiment performed in triplicate. The means were compared using Student's

t-test assuming equal variances and $p < 0.05$ was considered statistically significant.

Results

Cell culture

After a few days in a three dimensional alginate culture cells started to migrate from the alginate and adhered at the bottom of the culture flask, reaching confluence three days later. Emigrated chondrocytes exhibited a typical chondrogenic round shape and proliferated rapidly (data not shown).

Growth factors (IGF-I and TGF- β 1) inhibit IL-1 β -induced degenerative features and apoptosis in emigrated chondrocytes in monolayer culture

Chondrocytes in control monolayer cultures showed a typical flattened shape with small cytoplasmic processes, a large mostly euchromatic nucleus with nucleoli and a well structured cytoplasm (Fig. 1A). Treatment of chondrocyte cultures with 10 ng/ml IL-1 β for 24 hours led to degenerative changes, such as multiple vacuoles, swelling of rough ER, clustering of swollen mitochondria and degeneration of other cell organelles (Fig. 1B). After longer incubation periods (48 and 72 hours) more severe features of cellular degeneration were seen in response to IL-1 β treatment. These included areas of condensed heterochromatin in the cell nuclei and multiple and autophagic cytoplasmic vacuoles. The flattened monolayer chondrocytes became more and more rounded, lost their microvilli-like processes and became apoptotic (Fig. 1C-D). Chondrocytes that were pre-treated with IL-1 β (24 hours) and then co-treated with IL-1 β and IGF-I (100 ng/ml) or TGF- β 1 (100 ng/ml) for a additional 24 and 48 hours did not only show less severe cellular degeneration on the ultrastructural level (Fig. 1E-J), but further, in the presence of growth factors, the morphological degenerative features and mitochondrial swelling of the chondrocytes nearly disappeared (Fig. 1F,G,I,J). Combination treatment with both growth factors (IGF-I and TGF- β 1; each 10 ng/ml) clearly showed a time-dependent decrease in the morphological degenerative features, in mitochondrial swelling and apoptosis of the chondrocytes, indicating a potential synergism (Fig. 1K-M).

Down-regulation of collagen type II and β_1 -integrin-expression through IL-1 β on emigrated chondrocytes is blocked and revoked by growth factors

Chondrocytes, stimulated with IL-1 β alone showed down-regulation of the synthesis of collagen type II and β_1 -integrin in a time-dependent manner (Figs. 2, 3, I-II-III). Co-treatment of chondrocytes with IL-1 β and IGF-I or TGF- β 1 resulted not only in an inhibition of cytokine-induced effects on collagen type II- and β_1 -integrin-expression, but also, in the presence of growth factors,

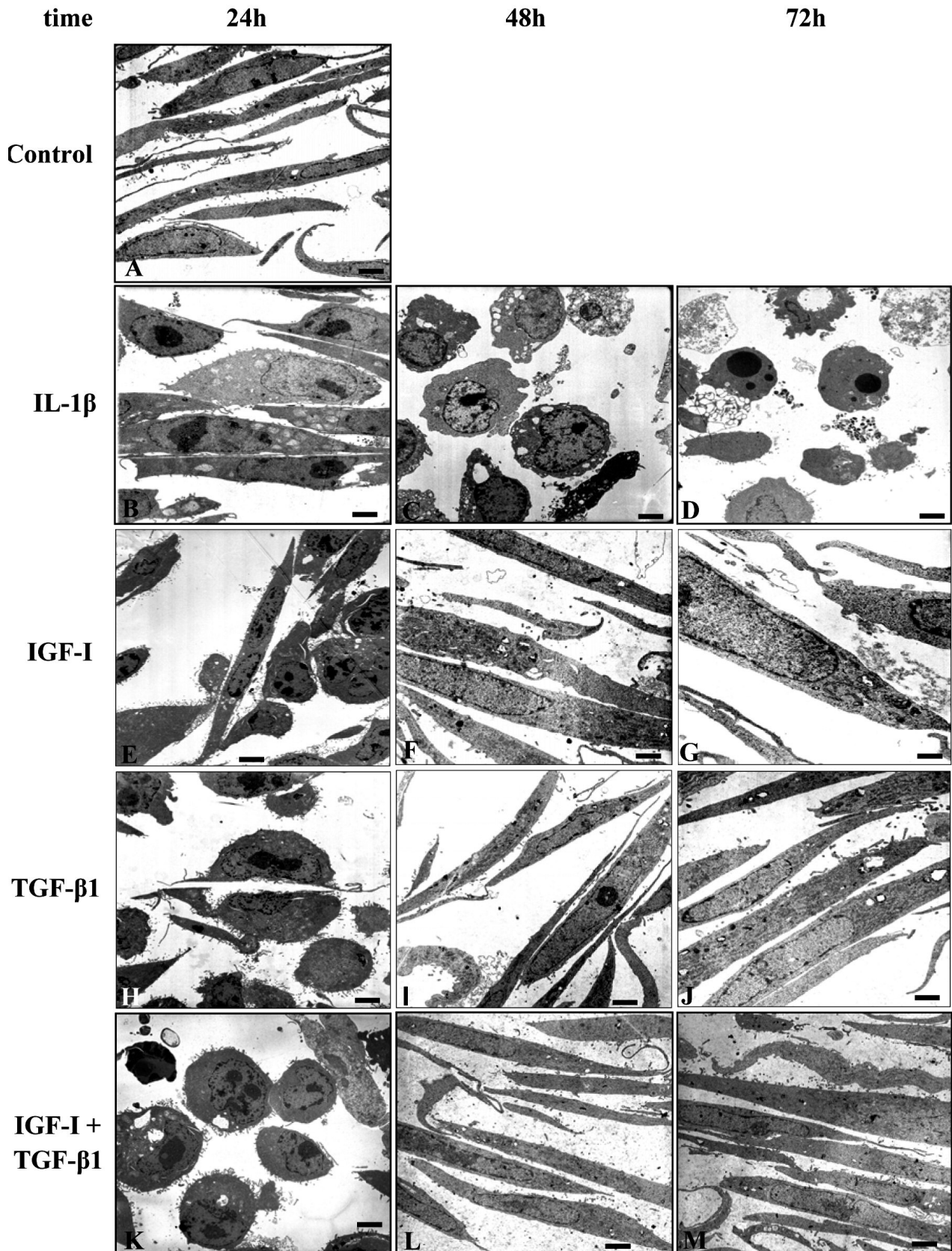


Fig. 1. IGF-I and TGF- β 1 inhibit IL-1 β -induced degenerative features and apoptosis in emigrated chondrocytes in monolayer culture. Chondrocytes, treated with IL-1 β for 24 hours (**B**), 48 hours (**C**) and 72 hours (**D**) exhibited characteristic features of degeneration. Chondrocytes that were pre-treated with IL-1 β for 24 hours (**E**, **H**, **K**) and then co-treated with IL-1 β and IGF-I for 24 hours (**F**) or 48 hours (**G**) or TGF- β 1 for 24 hours (**I**) or 48 hours (**J**) or both in combination for 24 hours (**L**) or 48 hours (**M**) showed less severe cell degeneration. In control cultures no ultrastructural changes were observed (**A**). x 4000; Bar: 1 μ M.

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collagen type II- and β_1 -integrin-production augmented significantly, reaching amounts of healthy, primary chondrocytes (Figs. 2, 3, I-II). Treatment of chondrocytes with both growth factors in combination resulted in a markedly enhanced stimulation of collagen type II- and β_1 -integrin-expression in comparison with

single growth factor treatment (Figs. 2, 3 III). Densitometric analysis of these results (Figs. 2, 3, I-II-III) showed that collagen type II- and β_1 -integrin-expression increased in growth factor-treated chondrocytes compared to IL-1 β -treated chondrocytes. Clearly, the combination of IGF-1 and TGF- β 1 showed

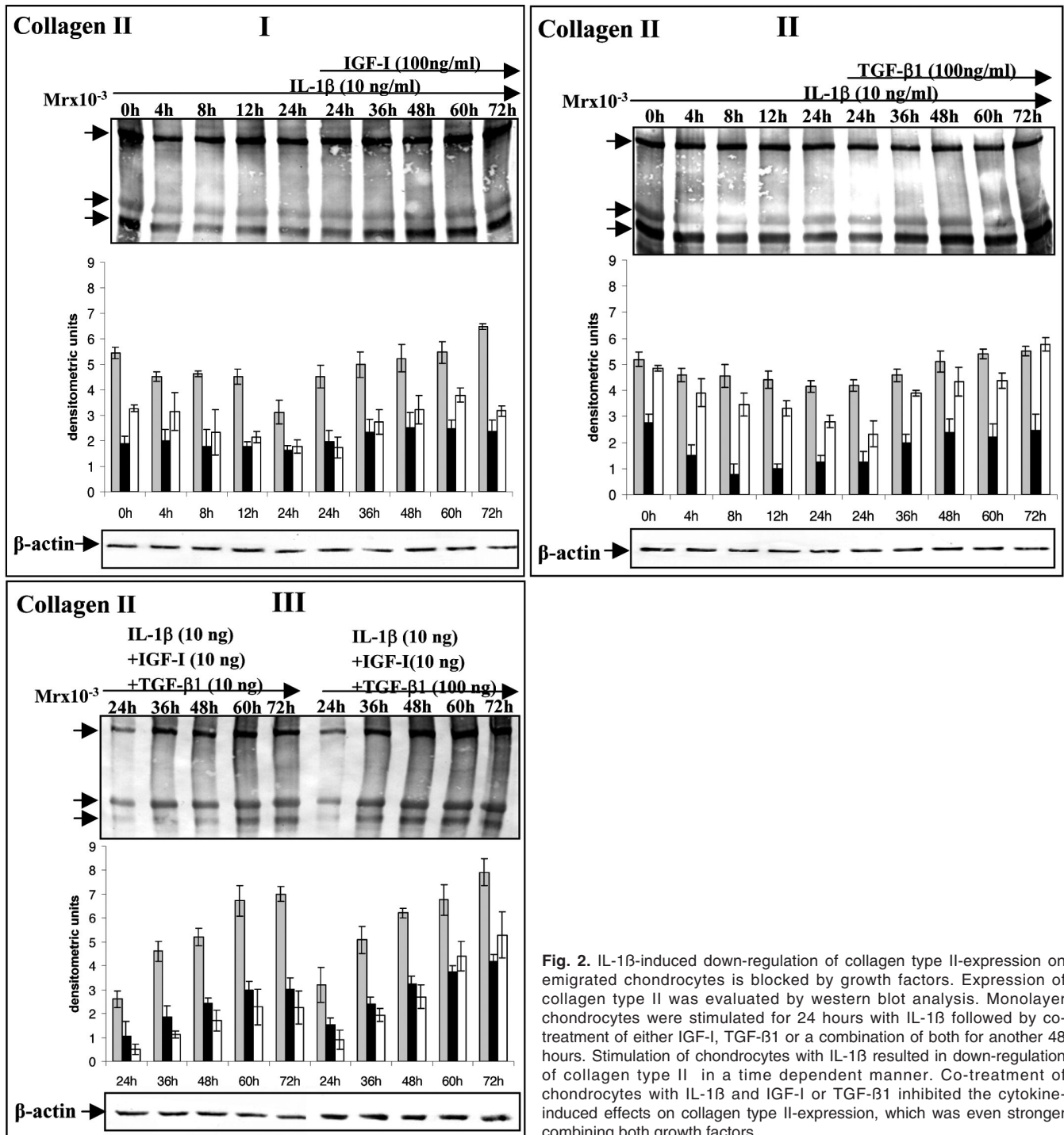


Fig. 2. IL-1 β -induced down-regulation of collagen type II-expression on emigrated chondrocytes is blocked by growth factors. Expression of collagen type II was evaluated by western blot analysis. Monolayer chondrocytes were stimulated for 24 hours with IL-1 β followed by co-treatment of either IGF-I, TGF- β 1 or a combination of both for another 48 hours. Stimulation of chondrocytes with IL-1 β resulted in down-regulation of collagen type II in a time dependent manner. Co-treatment of chondrocytes with IL-1 β and IGF-I or TGF- β 1 inhibited the cytokine-induced effects on collagen type II-expression, which was even stronger combining both growth factors.

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an additive effect on the expression of the above mentioned proteins. Co-treatment with IGF-I (10 ng/ml) and increased concentration of TGF-β1 (100 ng/ml) seemed to result in steady state β₁-integrin-expression. Synthesis of the house-keeping protein β-actin remained unaffected (Figs. 2, 3, I-II-III).

Growth factors suppress IL-1β-induced inhibition of Erk 1/2 and Sox9 in emigrated chondrocytes

Chondrocytes stimulated with IL-1β alone showed

down-regulation of the phosphorylated Erk 1/2 (42 kDa-band more than 44 kDa-band) in a time-dependent manner (Fig. 4, I-II-III). Co-treatment of chondrocytes with IL-1β and IGF-I or TGF-β1 resulted in an inhibition of IL-1β-induced effects on Erk 1/2-phosphorylation (Fig. 4, I-II). Combined treatment of chondrocytes with both growth factors resulted in a markedly stronger stimulation on the Erk 1/2-phosphorylation than each growth factor separately (Fig. 4, III). Erk 1 (44 kD) and Erk 2 (42 kD) show 90% sequence identity in mammals. Erk 2 is generally higher expressed in mammals.

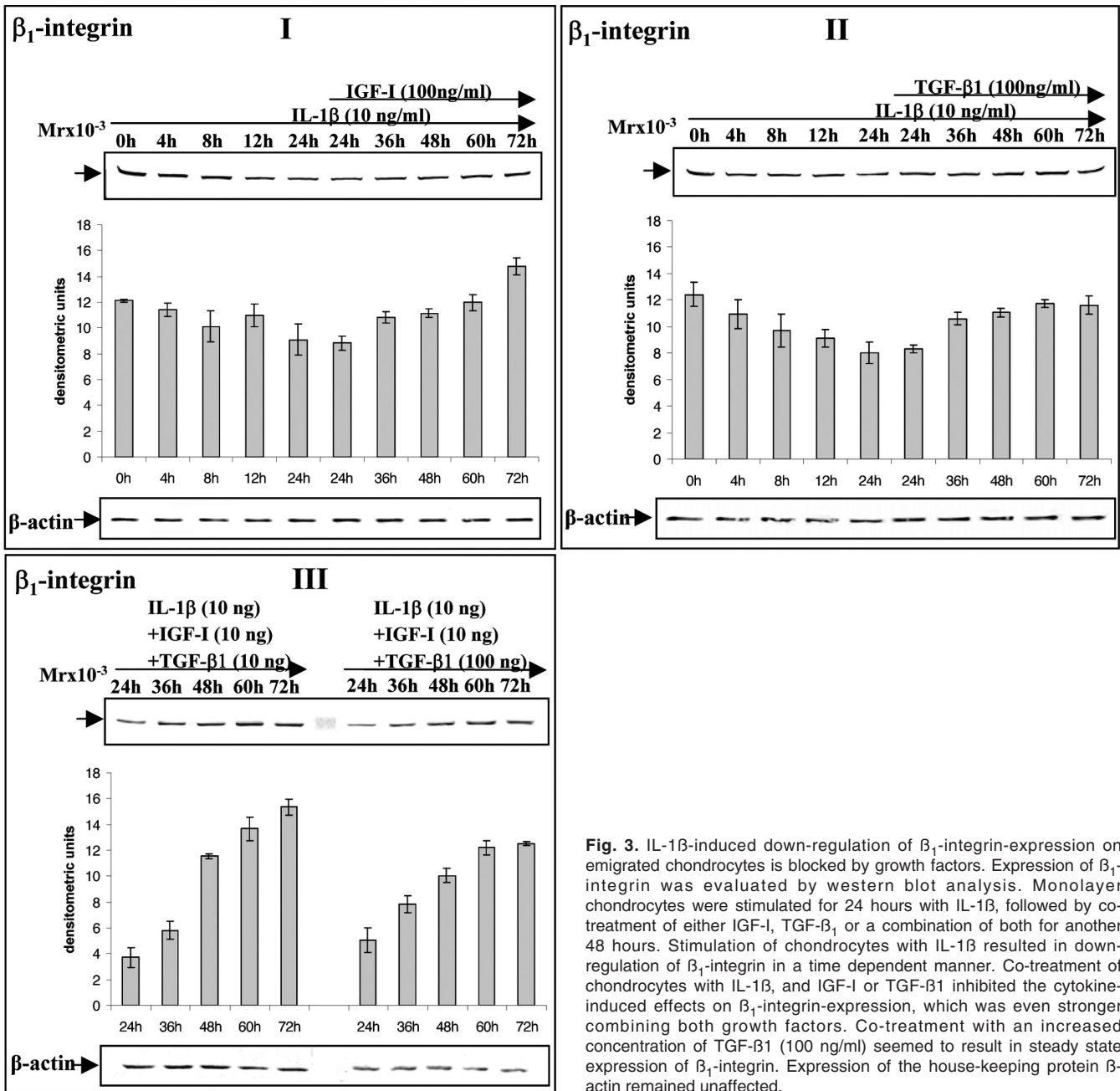


Fig. 3. IL-1β-induced down-regulation of β₁-integrin-expression on emigrated chondrocytes is blocked by growth factors. Expression of β₁-integrin was evaluated by western blot analysis. Monolayer chondrocytes were stimulated for 24 hours with IL-1β, followed by co-treatment of either IGF-I, TGF-β₁ or a combination of both for another 48 hours. Stimulation of chondrocytes with IL-1β resulted in down-regulation of β₁-integrin in a time dependent manner. Co-treatment of chondrocytes with IL-1β, and IGF-I or TGF-β₁ inhibited the cytokine-induced effects on β₁-integrin-expression, which was even stronger combining both growth factors. Co-treatment with an increased concentration of TGF-β₁ (100 ng/ml) seemed to result in steady state expression of β₁-integrin. Expression of the house-keeping protein β-actin remained unaffected.

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Therefore, as observed, the influence of combination treatment of IGF-I and TGF-β1 was better visibly in the 42 kDa-band. This points to an additive action of the combination treatment of IGF-I and TGF-β1. Densitometric analysis of the above presented results (Fig. 4, I-II-III) showed that Erk 1/2-phosphorylation increased in growth factor-treated chondrocytes compared to IL-1β-treated chondrocytes. The highest levels of Erk 1/2-phosphorylation were identified under

combined treatment with both growth factors. Synthesis of the house-keeping protein β-actin remained unaffected (Fig. 4, I-II-III).

Chondrocytes stimulated with IL-1β alone showed down-regulation of the synthesis of Sox9 in a time-dependent manner (Fig. 5, I-II-III). Co-treatment of chondrocytes with IL-1β and IGF-I or TGF-β1 resulted in an inhibition of cytokine-induced effects on Sox9 (Fig. 5, I-II). Combined treatment of emigrated

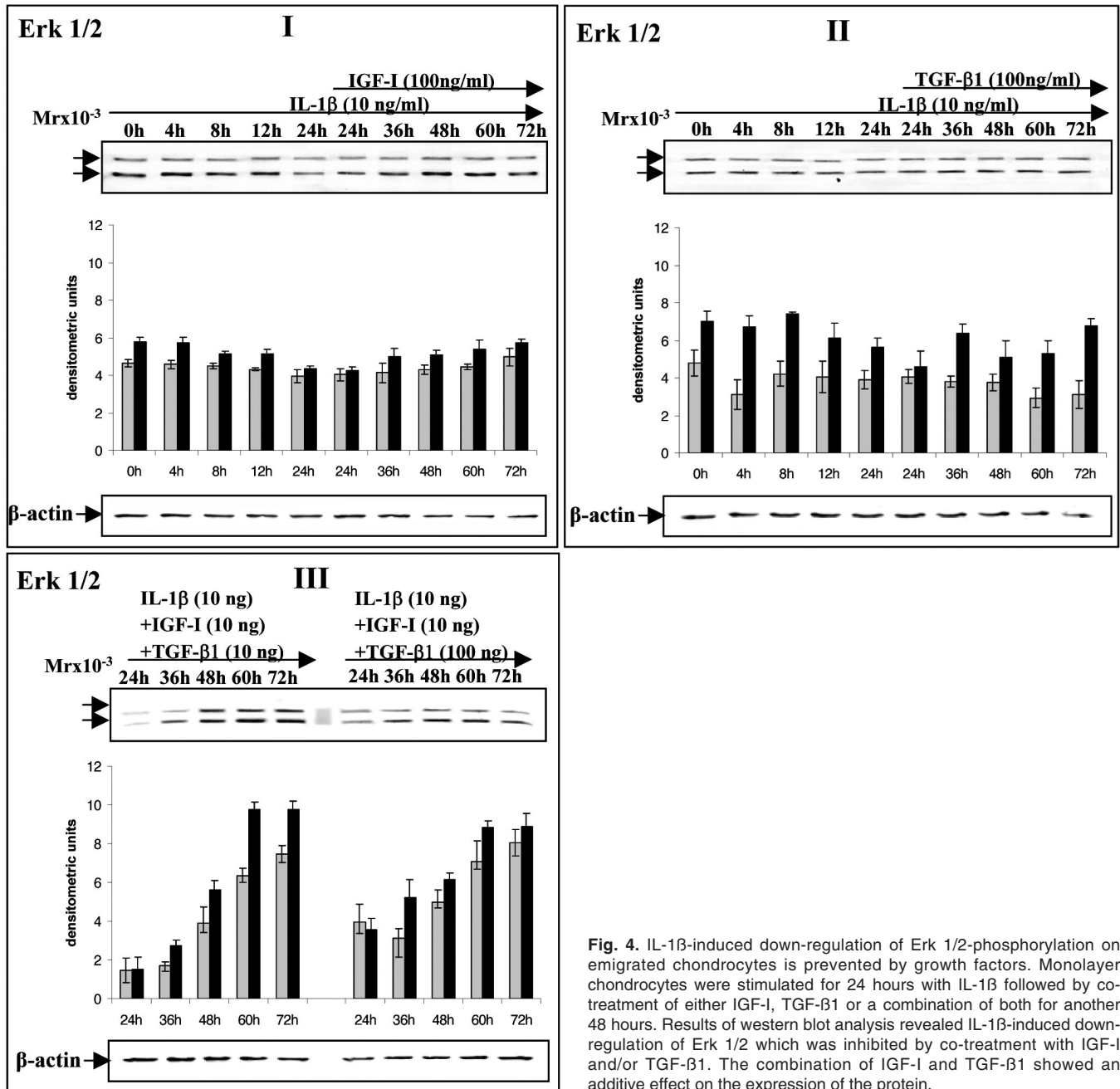


Fig. 4. IL-1β-induced down-regulation of Erk 1/2-phosphorylation on emigrated chondrocytes is prevented by growth factors. Monolayer chondrocytes were stimulated for 24 hours with IL-1β followed by co-treatment of either IGF-I, TGF-β1 or a combination of both for another 48 hours. Results of western blot analysis revealed IL-1β-induced down-regulation of Erk 1/2 which was inhibited by co-treatment with IGF-I and/or TGF-β1. The combination of IGF-I and TGF-β1 showed an additive effect on the expression of the protein.

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chondrocytes with both growth factors induced more expression of the Sox9 protein than each growth factor separately (Fig. 5, III). Densitometric analysis of a typical western blot experiment performed in triplicate (Fig. 5, I-II-III) showed that Sox9-expression increased in growth factor-treated chondrocytes compared to IL-1β-treated chondrocytes. The combination of the two growth factors stimulated the highest Sox9-synthesis, whereas in the presence of increased TGF-β1-

concentration (100 ng/ml) Sox9-expression seemed to reach a steady state. Synthesis of the house-keeping protein β-actin remained unaffected (Fig. 5, I-II-III).

Up-regulation of pro-inflammatory enzymes by IL-1β on emigrated chondrocytes is inhibited by growth factors

Chondrocytes stimulated with IL-1β alone showed up-regulation of the synthesis of COX-2 and MMP-13 in

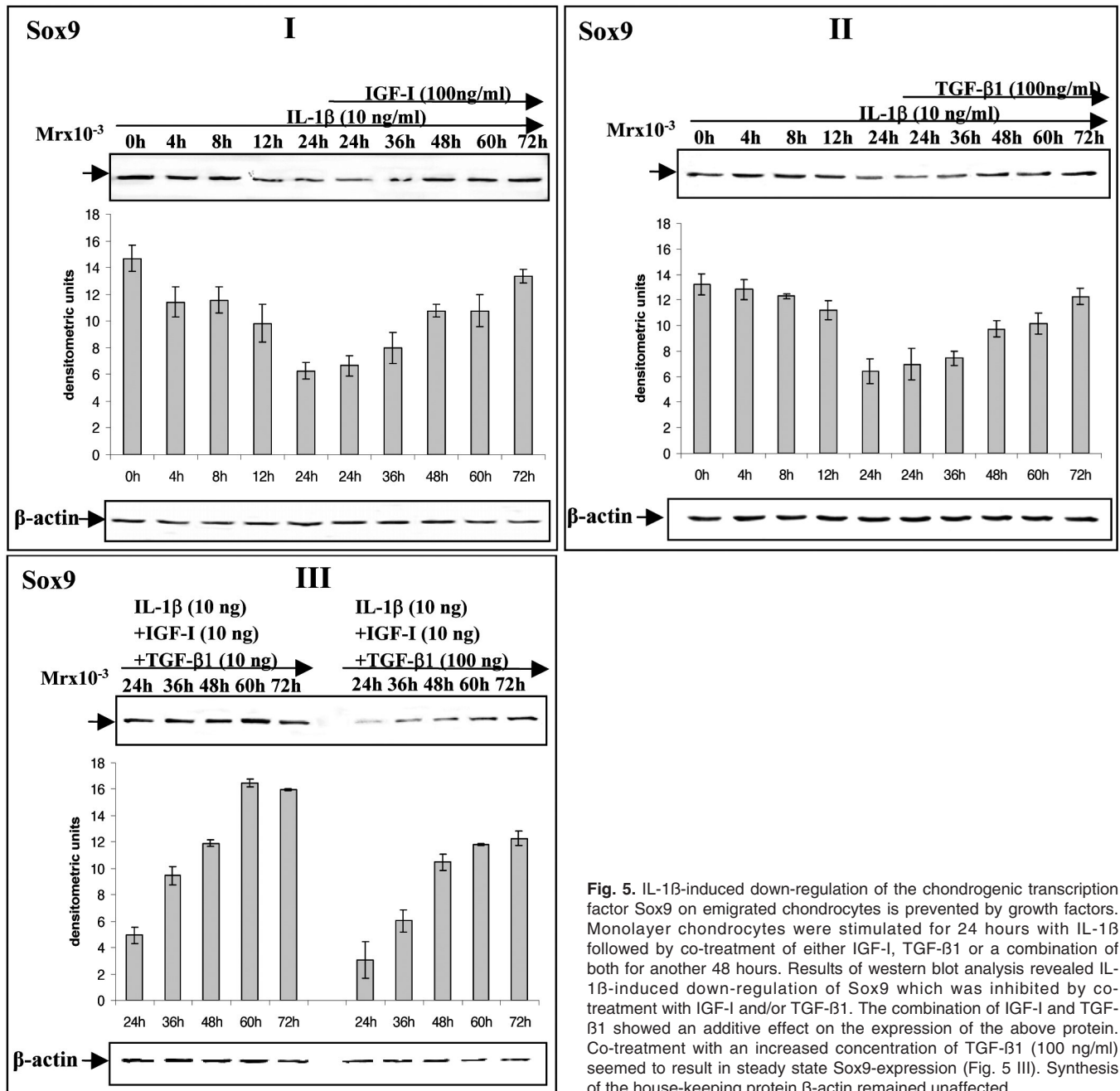


Fig. 5. IL-1β-induced down-regulation of the chondrogenic transcription factor Sox9 on emigrated chondrocytes is prevented by growth factors. Monolayer chondrocytes were stimulated for 24 hours with IL-1β followed by co-treatment of either IGF-I, TGF-β1 or a combination of both for another 48 hours. Results of western blot analysis revealed IL-1β-induced down-regulation of Sox9 which was inhibited by co-treatment with IGF-I and/or TGF-β1. The combination of IGF-I and TGF-β1 showed an additive effect on the expression of the above protein. Co-treatment with an increased concentration of TGF-β1 (100 ng/ml) seemed to result in steady state Sox9-expression (Fig. 5 III). Synthesis of the house-keeping protein β-actin remained unaffected.

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a time-dependent manner (Figs. 6, 7). Co-treatment of chondrocytes with IL-1β and IGF-1 or TGF-β1 resulted in an inhibition of cytokine-induced up-regulation of COX-2 and MMP-13 (Figs. 6, 7, I-II). Treatment of chondrocytes with both growth factors resulted in a stronger inhibition of COX-2- and MMP-13-expression compared to single growth factor treatment (Figs. 6, 7, III). Densitometric analysis of the above shown results (Figs. 6, 7) demonstrated that COX-2- and MMP-13-expression decreased in growth factor-treated cells

compared to IL-1β-treated chondrocytes. Combination of the two growth factors had the strongest inhibitory effect on the expression of the above mentioned proteins. Synthesis of the house-keeping protein β-actin remained unaffected (Figs. 6, 7).

Redifferentiation of dedifferentiated emigrated chondrocytes in high-density culture by growth factors

After seven days in high-density cultures,

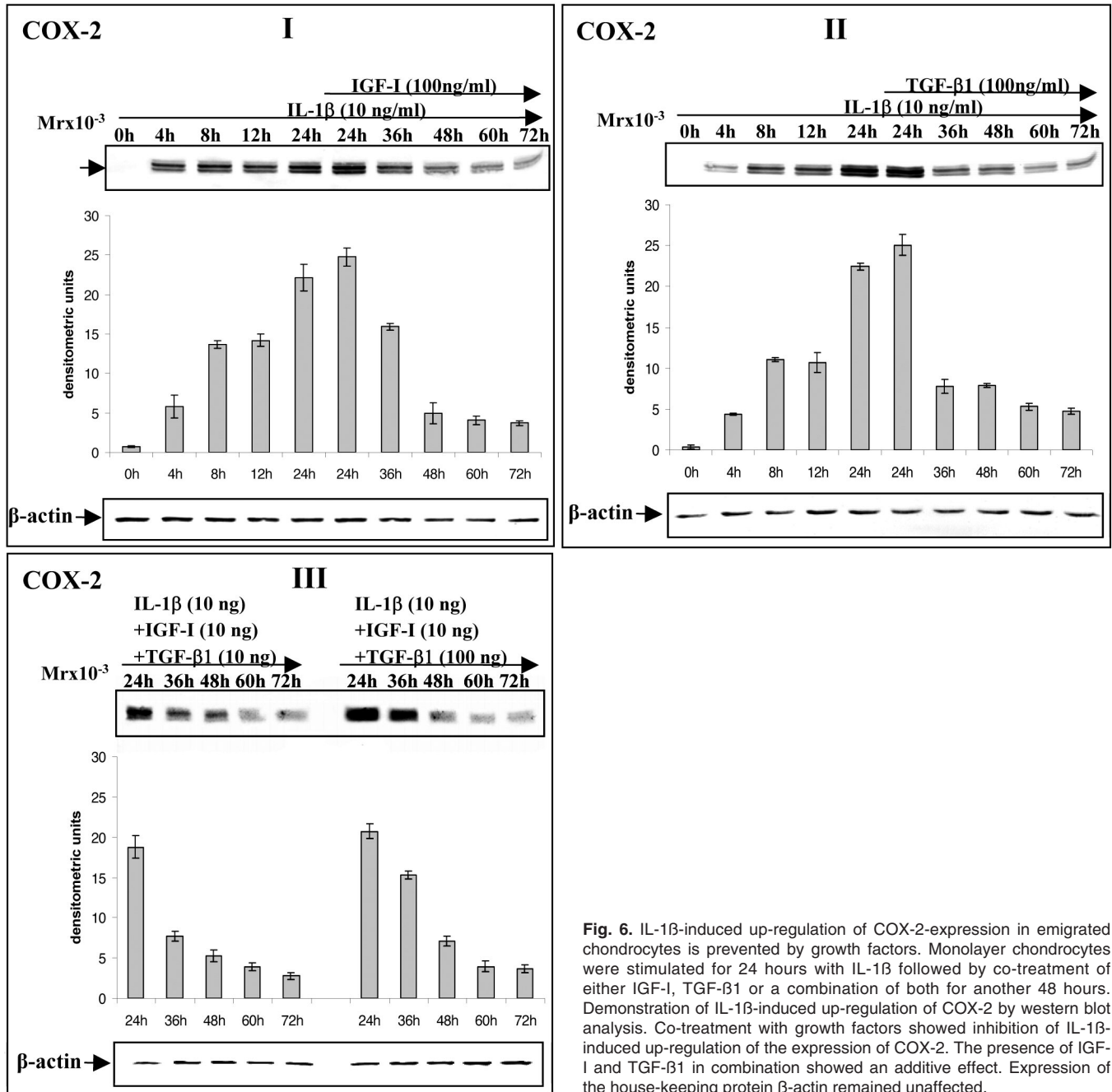


Fig. 6. IL-1β-induced up-regulation of COX-2-expression in emigrated chondrocytes is prevented by growth factors. Monolayer chondrocytes were stimulated for 24 hours with IL-1β followed by co-treatment of either IGF-I, TGF-β1 or a combination of both for another 48 hours. Demonstration of IL-1β-induced up-regulation of COX-2 by western blot analysis. Co-treatment with growth factors showed inhibition of IL-1β-induced up-regulation of the expression of COX-2. The presence of IGF-I and TGF-β1 in combination showed an additive effect. Expression of the house-keeping protein β-actin remained unaffected.

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dedifferentiated cells derived from monolayer cultures stimulated for 24 hours with IL-1β followed by co-treatment of either IGF-I, TGF-β1 or a combination of both for another 24 hours, had redifferentiated into chondrocytes similar to control cultures (Figs. 8A,C-E). Typically, these cells had a rounded morphology and contained a well-developed rough endoplasmic reticulum, a large Golgi apparatus, the usual complement of mitochondria, small vacuoles and granules. The cells had formed a matrix, which was closely attached to the

plasma membrane. This cartilaginous matrix consisted of fine collagen fibrils running singly and irregularly (Fig. 8A, C-E). Dedifferentiated cells derived from IL-1β-treated cultures did not redifferentiate into chondrocytes when recultivated in high-density cultures (Fig. 8B). They did not produce cartilage-specific matrix and underwent apoptosis, the characteristic morphological features of this process being membrane blebbing, disintegration of the nucleus, chromatin condensation along the nuclear envelope and the

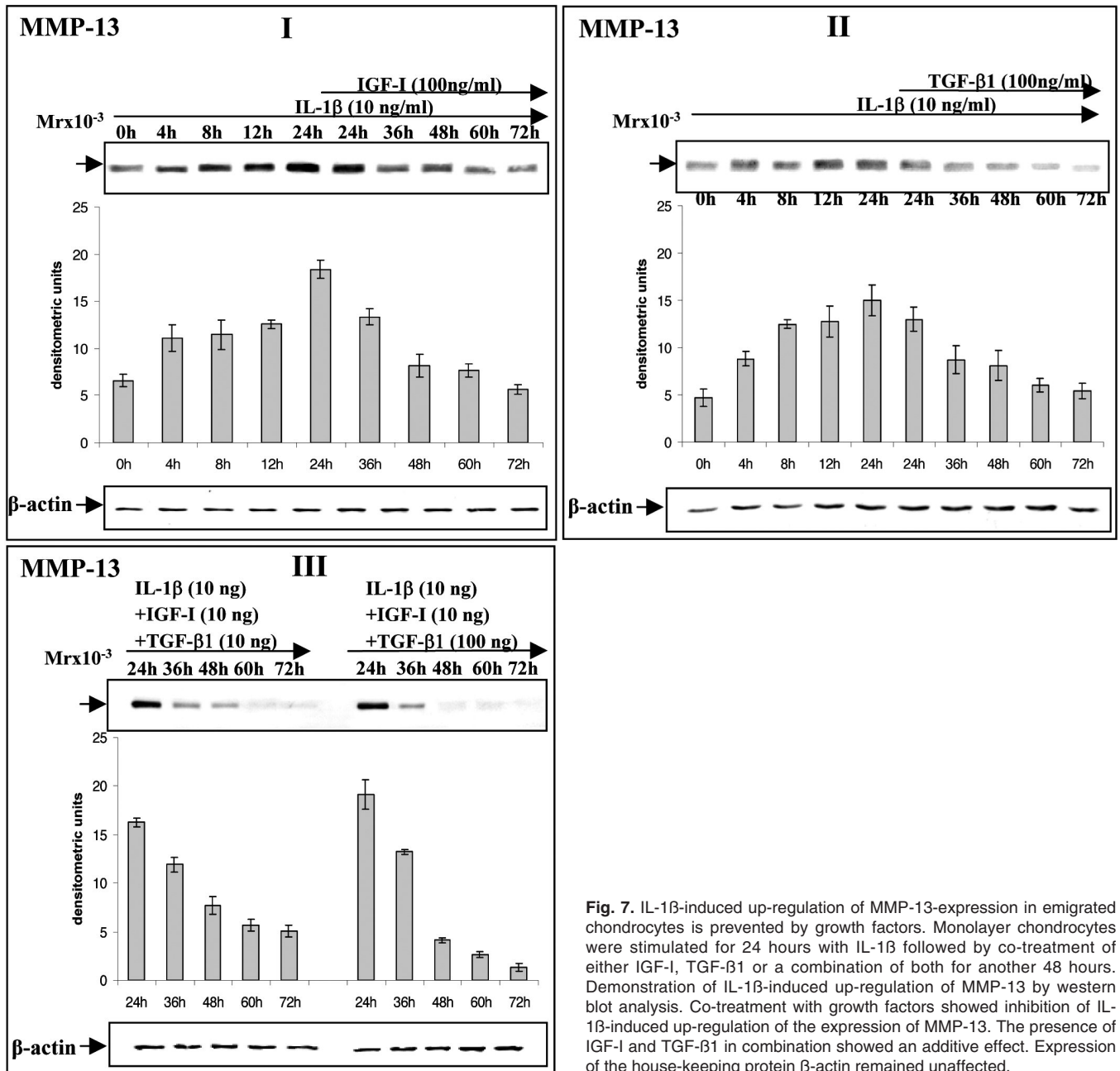


Fig. 7. IL-1β-induced up-regulation of MMP-13-expression in emigrated chondrocytes is prevented by growth factors. Monolayer chondrocytes were stimulated for 24 hours with IL-1β followed by co-treatment of either IGF-I, TGF-β1 or a combination of both for another 48 hours. Demonstration of IL-1β-induced up-regulation of MMP-13 by western blot analysis. Co-treatment with growth factors showed inhibition of IL-1β-induced up-regulation of the expression of MMP-13. The presence of IGF-I and TGF-β1 in combination showed an additive effect. Expression of the house-keeping protein β-actin remained unaffected.

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presence of apoptotic bodies (Fig. 8B).

Growth factors enhance the expression of collagen type II and β_1 -integrin during redifferentiation of dedifferentiated emigrated chondrocytes in high-density culture

Western blot investigations revealed high expressions of collagen type II (Fig. 9A) and of β_1 -integrin (Fig. 9B) in cells derived from control cultures (0, 12, 24 and 48 hours) in contrast to cultures treated with IL-1 β , which showed no or only low expression of collagen type II and β_1 -integrin (Fig. 9A,B). In the presence of IGF-I there was a high increase in the expression of both proteins. Further enhanced expression could be demonstrated after co-treatment with TGF- β 1 and even stronger expression using the combination of both growth factors. These results substantiate the morphological findings described by the electron microscopic demonstrations. Densitometric analysis of these results showed that collagen type II- and β_1 -integrin-expression increased in growth factor-treated chondrocytes compared to IL-1 β -treated chondrocytes after transfer into high-density culture. Clearly, the combination of IGF-I and TGF- β 1 showed an additive effect on the expression of the above-mentioned proteins. Synthesis of the house-keeping protein β -actin

remained unaffected (Fig. 9A,B).

Up-regulation of Erk 1/2 and Sox9 in redifferentiated emigrated chondrocytes by growth factors in high-density culture

Western blot analysis demonstrated IL-1 β -induced down-regulation of both MAPK members in contrast to control cells and co-treatment with growth factors (Fig. 9C,D). In the presence of IGF-I there was increased expression of Erk 1/2 and Sox9, which was enhanced by co-treatment with TGF- β 1. The strongest up-regulation of Erk 1/2 and Sox9 was observed in combination treatment of IGF-I and TGF- β 1 (Fig. 9C,D). Densitometric analysis of the above presented results demonstrated that Erk 1/2- and Sox9-expression increased in growth factor-treated chondrocytes compared to IL-1 β -treated chondrocytes after transfer to high-density culture. A combination of both growth factors demonstrated an additive effect on the expression of Erk 1/2 and Sox9. Synthesis of the house-keeping protein β -actin remained unaffected (Fig. 9C-D).

IL-1 β -induced apoptosis in emigrated chondrocytes is blocked by growth factors in high-density culture

As shown in Fig. 9E there was a high increase of

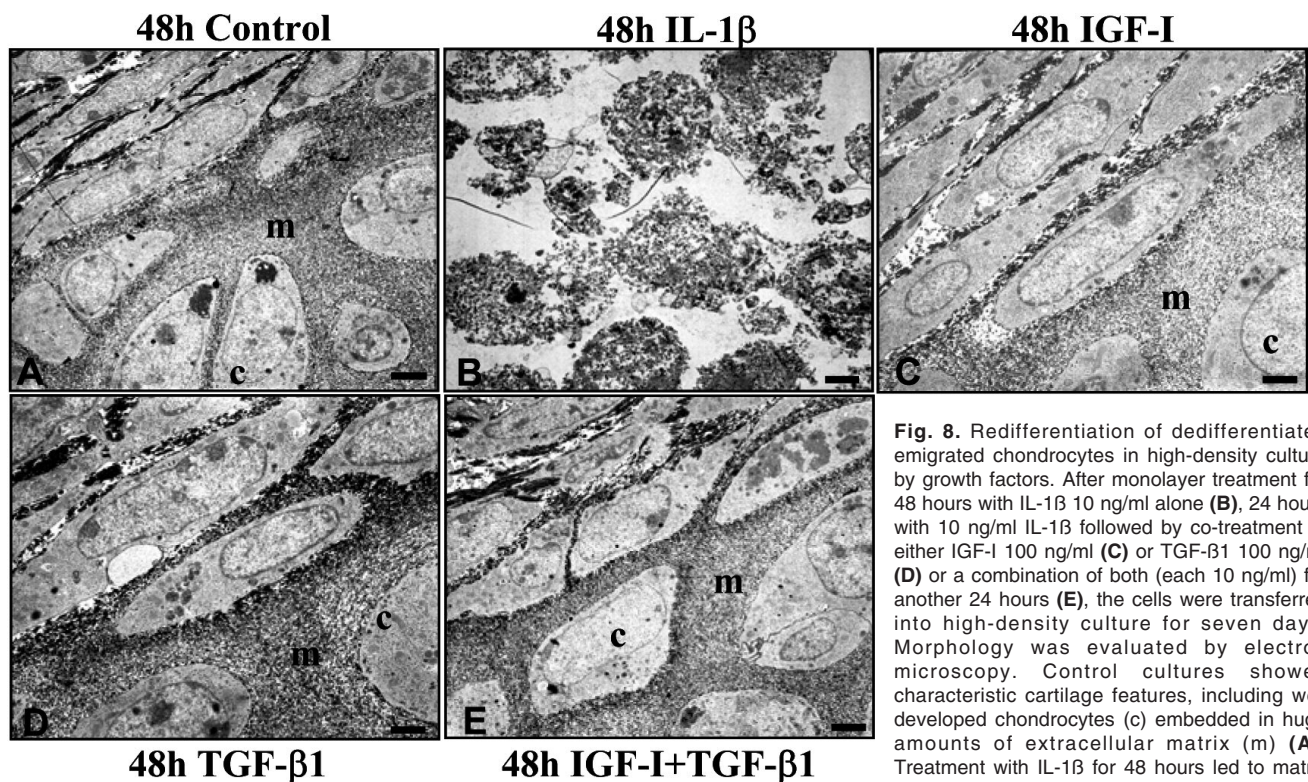


Fig. 8. Redifferentiation of dedifferentiated emigrated chondrocytes in high-density culture by growth factors. After monolayer treatment for 48 hours with IL-1 β 10 ng/ml alone (B), 24 hours with 10 ng/ml IL-1 β followed by co-treatment of either IGF-I 100 ng/ml (C) or TGF- β 1 100 ng/ml (D) or a combination of both (each 10 ng/ml) for another 24 hours (E), the cells were transferred into high-density culture for seven days. Morphology was evaluated by electron microscopy. Control cultures showed characteristic cartilage features, including well developed chondrocytes (c) embedded in huge amounts of extracellular matrix (m) (A). Treatment with IL-1 β for 48 hours led to matrix breakdown and even cell lysis (B). Application of IGF-I (C), TGF- β 1 (D) or both in combination (E) resulted in marked improvement of chondrocyte phenotype and even in the formation of cartilage nodules. Very dense formation of extracellular matrix (m) surrounding well developed chondrocytes (c) was observed. x 4000; Bars: 1 μ m.

IGF-I (C), TGF- β 1 (D) or both in combination (E) resulted in marked improvement of chondrocyte phenotype and even in the formation of cartilage nodules. Very dense formation of extracellular matrix (m) surrounding well developed chondrocytes (c) was observed. x 4000; Bars: 1 μ m.

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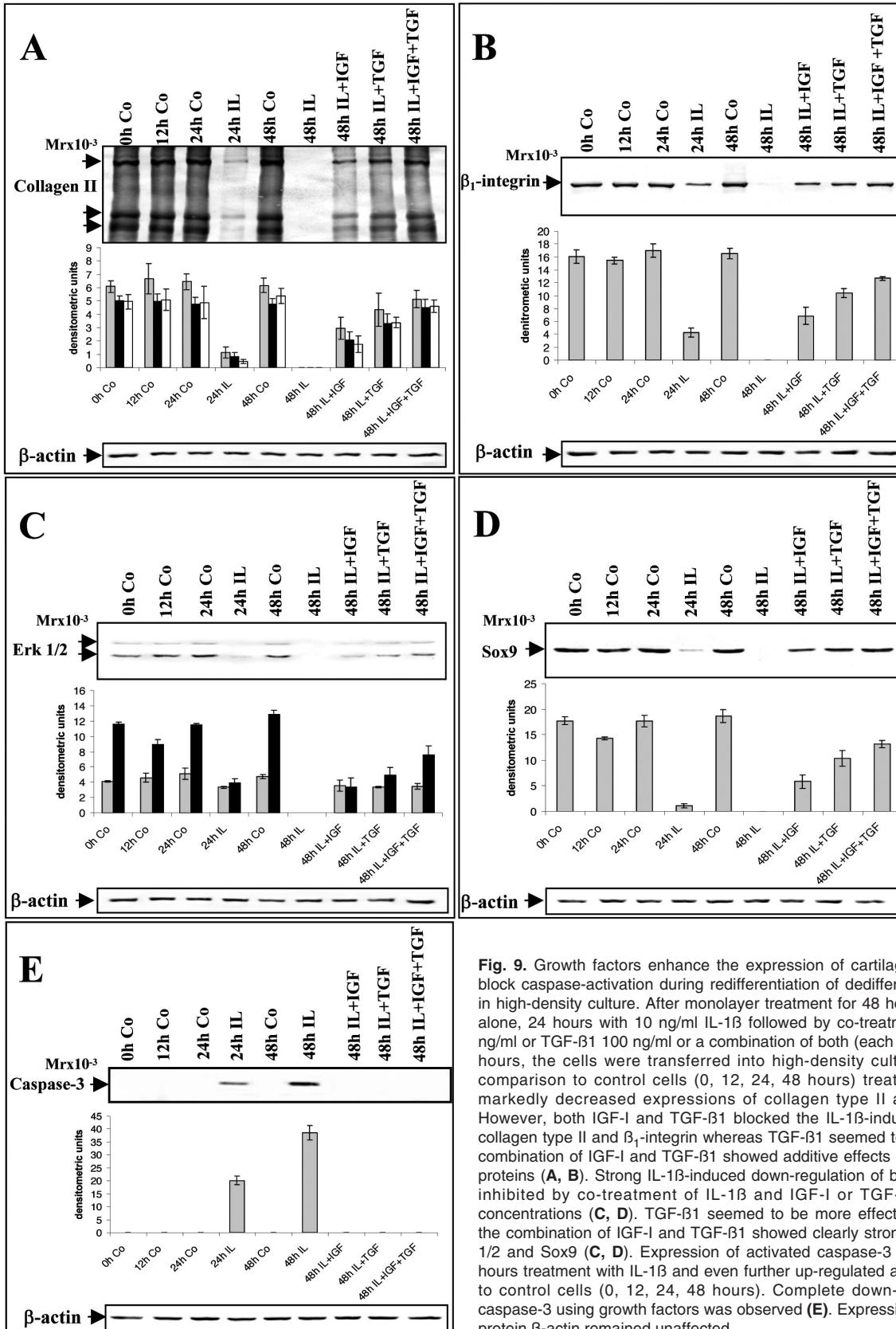


Fig. 9. Growth factors enhance the expression of cartilage-specific proteins and block caspase-activation during redifferentiation of dedifferentiated emigrated cells in high-density culture. After monolayer treatment for 48 hours with IL-1β 10 ng/ml alone, 24 hours with 10 ng/ml IL-1β followed by co-treatment of either IGF-I 100 ng/ml or TGF-β1 100 ng/ml or a combination of both (each 10 ng/ml) for another 24 hours, the cells were transferred into high-density culture for seven days. In comparison to control cells (0, 12, 24, 48 hours) treatment with IL-1β led to markedly decreased expressions of collagen type II and β₁-integrin (**A**, **B**). However, both IGF-I and TGF-β1 blocked the IL-1β-induced down-regulation of collagen type II and β₁-integrin whereas TGF-β1 seemed to be more effective. The combination of IGF-I and TGF-β1 showed additive effects on the above mentioned proteins (**A**, **B**). Strong IL-1β-induced down-regulation of both Erk 1/2 and Sox9 is inhibited by co-treatment of IL-1β and IGF-I or TGF-β1 or both at various concentrations (**C**, **D**). TGF-β1 seemed to be more effective than IGF-I, however the combination of IGF-I and TGF-β1 showed clearly stronger up-regulation of Erk 1/2 and Sox9 (**C**, **D**). Expression of activated caspase-3 is up-regulated after 24 hours treatment with IL-1β and even further up-regulated after 48 hours in contrast to control cells (0, 12, 24, 48 hours). Complete down-regulation of activated caspase-3 using growth factors was observed (**E**). Expression of the house-keeping protein β-actin remained unaffected.

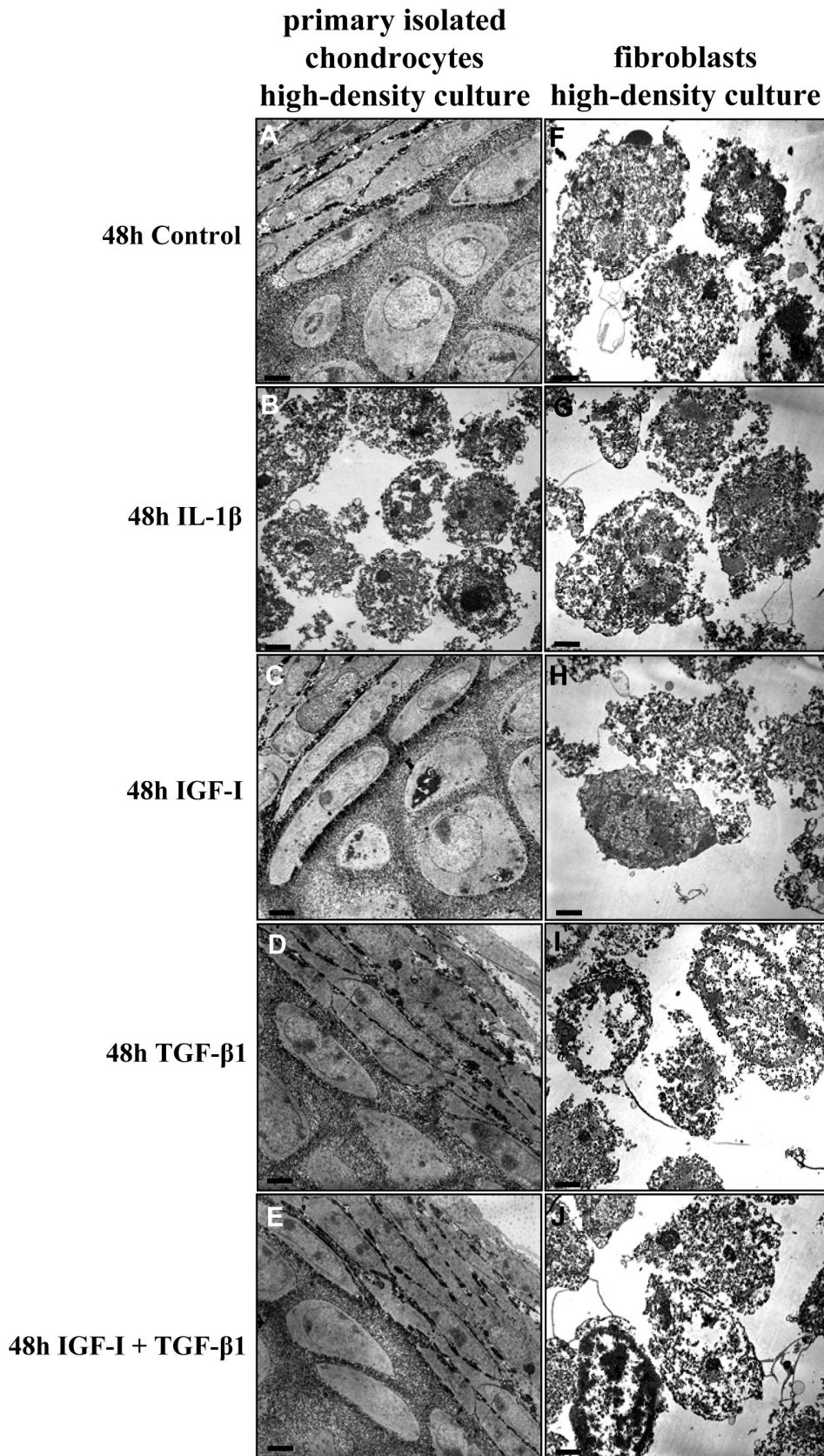


Fig. 10. Cultivation of primary isolated chondrocytes and fibroblasts in high-density culture. Primary isolated chondrocytes and fibroblasts were stimulated with IL-1 β (10 ng/ml) alone for 24 hours in monolayer, before co-treatment with IGF-I or TGF- β 1 (each 100 ng/ml) or both in combination (each 10 ng/ml) or treated only with IL-1 β for an additional 24 hours. Afterwards the cells were transferred to high-density culture for seven days. Morphology was evaluated by transmission electron microscopy. Control cultures of primary chondrocytes showed well developed cartilage nodules (A). Treatment with IL-1 β resulted in cell destruction after 48 hours (B). However, after co-treatment of primary chondrocytes with IGF-I (C) or TGF- β 1 (D) or both in combination (E) there were well defined cartilage nodules. In contrast, in fibroblast high-density culture there was no difference between treatment with IL-1 β or growth factors. In both cases treatment resulted in cell lysis (F-J). x 4000; Bars: 1 μ m.

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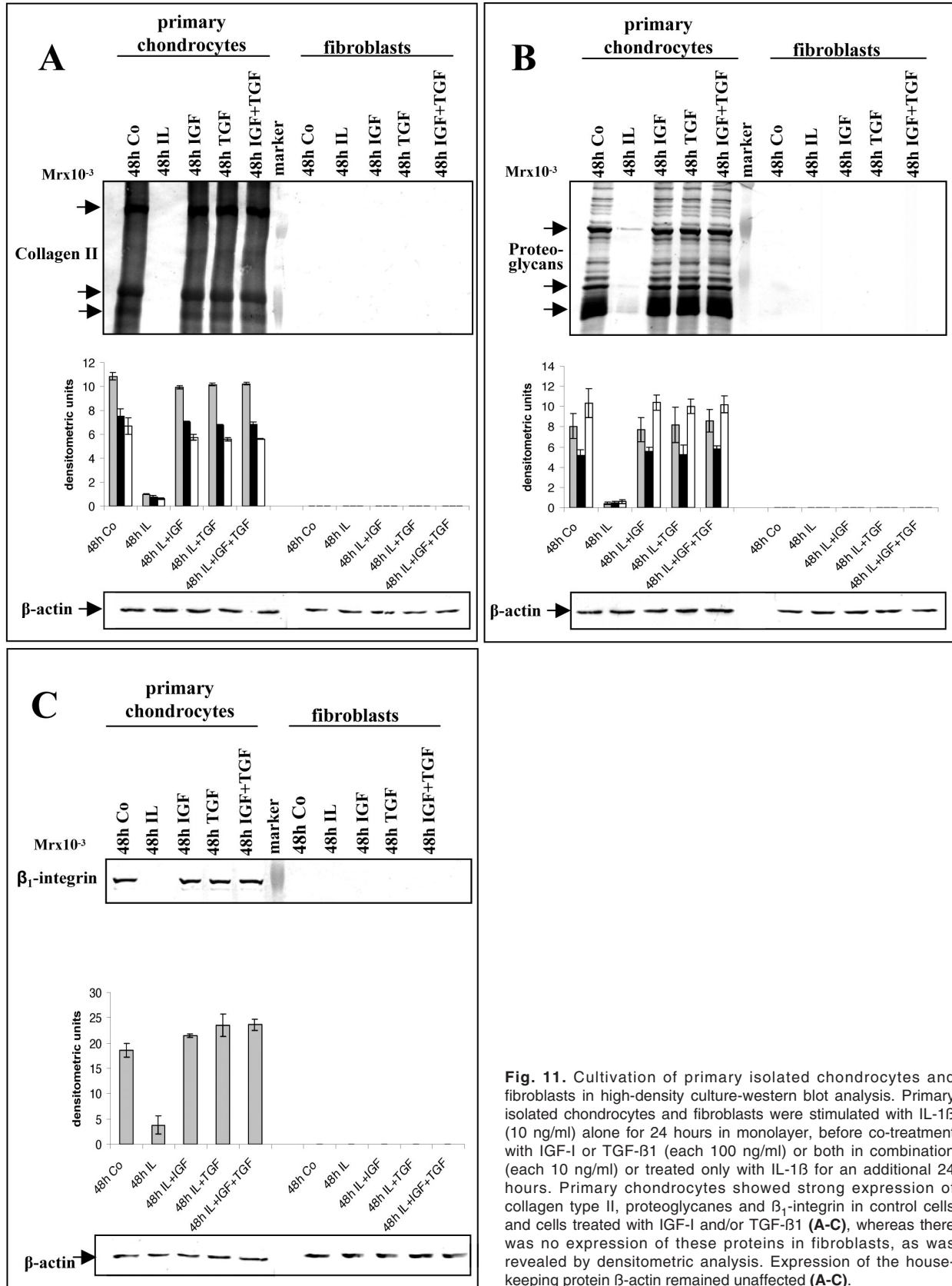


Fig. 11. Cultivation of primary isolated chondrocytes and fibroblasts in high-density culture-western blot analysis. Primary isolated chondrocytes and fibroblasts were stimulated with IL-1β (10 ng/ml) alone for 24 hours in monolayer, before co-treatment with IGF-I or TGF-β1 (each 100 ng/ml) or both in combination (each 10 ng/ml) or treated only with IL-1β for an additional 24 hours. Primary chondrocytes showed strong expression of collagen type II, proteoglycans and β₁-integrin in control cells and cells treated with IGF-I and/or TGF-β1 (A-C), whereas there was no expression of these proteins in fibroblasts, as was revealed by densitometric analysis. Expression of the house-keeping protein β-actin remained unaffected (A-C).

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activated caspase-3 after treatment with IL-1 β (10 ng/ml) for 24 hours and even higher expression after 48 hours. In the presence of IGF-I or TGF- β 1 or both in combination there was complete down-regulation of activated caspase-3. Densitometric analysis of these results showed increased expression of activated caspase-3 in IL- β -treated chondrocytes compared to control chondrocytes and chondrocytes co-treated with IGF-I and/or TGF- β 1 after transfer to high-density culture. Synthesis of the house-keeping protein β -actin remained unaffected (Fig. 9E).

Cultivation of primary isolated chondrocytes and fibroblasts in high-density culture

To test the results performed with emigrated cells from the alginate system, the same investigations were performed in high-density culture with either primary chondrocytes as a positive control or with fibroblasts to demonstrate negative control. As shown in Fig. 10, control cultures of primary chondrocytes showed well developed cartilage nodules. The cells appear as viable chondrocytes exhibiting characteristic morphological features, and they formed a strong and regular fibrillar extracellular matrix (Fig. 10A). In contrast, primary chondrocytes underwent apoptosis when treated with IL-1 β (10 ng/ml) for 48 hours (Fig. 10B). However, treatment with growth factors resulted in well developed cartilage nodules with viable cells and well developed and organized cell organelles. The cells formed a dense and regular extracellular matrix construct (Fig. 10C-E). In opposite, high-density cultures of fibroblasts were completely destroyed in the presence of IL-1 β (Fig. 10G), as well as after co-treatment with growth factors (Fig. 10H-J).

Western blot analysis using antibodies against collagen type II, proteoglycans and β ₁-integrin revealed strong expression in control and growth factor-treated primary chondrocytes, but only low expression after treatment with IL-1 β (Fig. 11A-C). In contrast, the expression of the above proteins was neither detectable in growth factor- nor in IL-1 β -treated fibroblasts (Fig. 11A-C). Densitometric analysis of the above obtained results confirmed these results. Synthesis of the house-keeping protein β -actin remained unaffected (Fig. 11A-C).

Discussion

This is a continuing study of Shakibaei et al. (2006) that compares the effect of IGF-I, TGF- β 1 or their combination on human articular chondrocytes *in vitro*. The data presented in this study provide convincing molecular evidence to support the hypothesis that IGF-I and TGF- β 1 suppress IL-1 β -induced apoptosis and cartilage degradation by up-regulation of the MAPK and Sox9-regulated signalling pathway.

We have made the following novel observations: (1) IL-1 β -induced morphological alterations, swollen mitochondria, dilated endoplasmic reticulum and apoptosis were abolished by co-treatment with IGF-I

or/and TGF- β 1. (2) IGF-I or TGF- β 1 inhibited the IL-1 β -mediated suppression of key extracellular matrix components, β ₁-integrin and integrin signalling proteins. (3) IGF-I or TGF- β 1 were able to antagonize the IL-1 β -dependent up-regulation of MMP-13 and COX-2. (4) Indeed, IGF-I and/or TGF- β 1 stabilize the chondrogenic potential in monolayer cultures by the Erk1/2 and MAPK signalling pathway and Sox9-stimulation, strongly suggesting that these effects are regulated by the integrin signalling pathway. (5) Interestingly, these effects were significantly higher in cells treated with both growth factors than with single treatment. (6) Furthermore, the redifferentiation capability of dedifferentiated monolayer chondrocytes in high-density culture was stimulated by growth factors and this capacity was clearly higher in the presence of both growth factors.

Primary isolated chondrocytes cultured in alginate beads and then emigrated from alginate beads present a pure chondrocyte population when introduced into monolayer culture (Schulze-Tanzil et al., 2004; Shakibaei et al., 2008). Indeed, production of cartilage-specific markers by emigrated cells could be shown in the present study and in previous investigations (Schulze-Tanzil et al., 2002, 2004). Therefore we selected these emigrated cells as a representative chondrocyte subset of cells for our study.

Growth factors stabilize the chondrogenic potential in IL-1 β -treated dedifferentiated chondrocytes in vitro

Exposing chondrocytes to the pro-inflammatory cytokine IL-1 β in monolayer culture led to phenotypical dedifferentiation, finally resulting in apoptotic cell death and even cell lysis after 72 hours. Indeed, a set of catabolic cytokines, including IL-1 β and TNF- α , are known to play an essential role in the pathogenesis of OA (Fernandes et al., 2002). For this reason, strategies for inhibiting or antagonizing this process need to be developed, or rather, must be improved. In the Autologous Chondrocyte Implantation (ACI) technique it is a major challenge to obtain sufficient quantities of differentiated chondrocytes to cover cartilage lesions and at the same time avoid irreversible dedifferentiation of these cells during the expansion phase in monolayer culture (Peterson et al., 2002). Recently, several investigators have reported on the beneficial effects of various growth factors such as FGF-2, IGF-I or TGF- β on chondrocyte differentiation or redifferentiation in culture (Martin et al., 1999; Jakob et al., 2001; Mandl et al., 2002; Barbero et al., 2003; Darling and Athanasiou, 2005). Indeed, we can demonstrate in this study the positive effects of IGF-I and/or TGF- β 1 on the maintenance of the chondrogenic potential in chondrocytes *in vitro*.

We found that IGF-I, similarly to TGF- β 1, stimulates the chondrogenic potential of IL-1 β -treated dedifferentiated chondrocytes, stabilizing the chondrocyte phenotype and stimulating proliferation, collagen type II- and β ₁-integrin-expression. Stimulation

of the signalling pathway by integrins leads to activation of the Ras-MAPkinase signalling pathway in chondrocytes. We also found that IL-1 β -induced down-regulation of the MAPKinase members Erk 1/2 and the master chondrogenic transcription factor Sox9 were inhibited by IGF-I or TGF- β 1. Moreover, the combination of IGF-I and TGF- β 1 showed an additive effect on the expression of the above mentioned proteins.

Cell-cell contact and cell-matrix interactions are essential and stimulate chondrocyte differentiation and survival (Yang et al., 1997; Shakibaei et al., 2001). β_1 -integrins as adhesion and signalling molecules play an important role in cell-matrix interactions in tissues, and inhibition of such interactions by antibodies in high-density cultures leads to irreversible dedifferentiation and apoptosis of chondrocytes (Shakibaei, 1998). Furthermore, the IGF-I-receptor has been shown before in our laboratories to cooperate with β_1 -integrins in chondrocytes and this interaction induces the MAPK pathway (Shakibaei et al., 1999), an important signalling cascade for chondrocyte differentiation and survival. Indeed, several investigators have already shown that the activation of key signalling proteins of the MAPK pathway increases Sox9 in chondrocytes and this process is improved by different growth factors (Schulze-Tanzil et al., 2004; Shakibaei et al., 2006). Moreover, blockade of the MAPK signal transduction pathway leads to chondrocyte apoptosis and inhibits the expression of Sox9 (Murakami et al., 2000; Shakibaei et al., 2001; Schulze-Tanzil et al., 2004). Sox9 is well known as the chondrogenic transcription factor for chondrocyte differentiation and expression of chondrocyte-specific anabolic genes, such as type II collagen and aggrecan (de Crombrugge et al., 2000; Kulyk et al., 2000). However, cartilage cells from OA express lower amounts of Sox9 compared to healthy cartilage cells (Aigner et al., 2003). This might be one explanation of how growth factor (IGF-I and TGF- β 1) treatment stabilizes the chondrogenic potential and chondrocyte survival via integrin/MAPK signalling in this system.

The detailed mechanism of the growth factor, influence has not been fully elucidated but several explanations for these positive effects can be discussed. Growth factors might increase the number of the metabolically stimulated cells (Loeser et al., 2003); a single growth factor might activate and trigger the signalling pathway in chondrocytes and a combination of growth factors may activate pathways that are not affected by a single growth factor and where a synergistic effect on cell differentiation and survival could be found (Yaeger et al., 1997; Tsukazaki et al., 1994; Im et al., 2003).

IGF-I and TGF- β 1 block the up-regulation of the pro-inflammatory enzymes COX-2 and MMP-13 in chondrocyte monolayer cultures

Moreover, we also found that IL-1 β -induced apoptosis and expression of pro-inflammatory enzymes and mediators, like COX-2 and MMP-13, were inhibited

by IGF-I and/or TGF- β 1. Furthermore, we observed that the combined treatment of IGF-I and TGF- β 1 had a stronger inhibitory effect on the expression of COX-2 and MMP-13 than each growth factor separately. Indeed, cytokines are potent stimulators for the *de novo synthesis* of catabolic enzymes such as MMPs, which in cartilage are responsible for excessive cartilage matrix degradation in Osteoarthritis (Robbins et al., 2000; Mort and Billington, 2001; Fernandes et al., 2002; Largo et al., 2003). Moreover, it has been reported and demonstrated that cytokines induced the MMP-13 gene during OA, and that the inhibition of this signalling was beneficial for reducing cartilage degradation in arthritis (Liacini et al., 2003). In healthy cartilage, there exists a balance between MMP-activity and their inhibitors, the tissue inhibitors of MMPs (known as TIMPs), and this is precisely regulated. However, in OA this fine balance between synthesis and degradation is disrupted, resulting in an increased activity of MMPs and therefore excessive extracellular matrix degradation and cartilage destruction (Gowen et al., 1984). Furthermore, other pro-inflammatory mediators were inhibited by growth factor treatment such as COX-2, which leads to prostaglandin E₂ (PGE₂)-production and pain (Sakai et al., 2001; Largo et al., 2003). COX-2 is an important mediator of inflammation and induces joint damage in OA and other osteoarticular disorders (Gowen et al., 1984). Moreover, COX-2-synthesis is induced by the cytokines IL-1 β and TNF- α (Sakai et al., 2001; Largo et al., 2003). We were able to demonstrate in this study that IGF-I or TGF- β 1 could inhibit IL-1 β -stimulated synthesis of COX-2. The inhibitory effect was more abundant in the combination treatment of both growth factors.

Growth factors inhibit IL-1 β -induced dedifferentiation and cell death in high-density cultures

Finally, to test whether the cells from monolayer cultures redifferentiate into chondrocytes, the emigrated cells treated with or without growth factors were transferred to high-density cultures. We found that the IL-1 β -treated emigrated cells from monolayer cultures co-treated with growth factors redifferentiated into chondrocytes after transfer to high-density culture, produced cartilage-specific matrix, i.e. collagen type II, integrin adhesion and signalling molecules, activated the MAPK pathway and the chondrogenic transcription factor Sox9. Furthermore, the expression of the pro-apoptotic marker (activated caspase-3) was inhibited in with IL-1 β and growth factors co-treated emigrated cells. We further found that the combined treatment with IGF-I and TGF- β 1 had a stronger inhibitory effect on caspase-3 expression than each growth factor separately.

To confirm the redifferentiation capacity of emigrated cells from alginate beads to cartilage development in high-density cultures, we performed the same experiments with primary isolated chondrocytes (positive control) and fibroblasts (negative control). Fibroblast cells, as opposed to primary chondrocytes and

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emigrated chondrocytes from alginate, were not able to survive in high-density cultures. These cells underwent cell death and did not produce any cartilage-specific markers in the presence or absence of growth factors.

It has been reported that growth factors such as FGF-2 and TGF- β can promote (re)differentiation of dedifferentiated chondrocytes in high-density cultures (Martin et al., 1999; Barbero et al., 2003) and increase the expression of Sox9 (Enomoto-Iwamoto et al., 1998; Zehentner et al., 1999). Furthermore, expression and activation of the Sox9 gene is inhibited by cytokines like IL-1 β and TNF- α , and this process seems to be blocked through growth factors mediated by the MAPK pathway (de Crombrugge et al., 2000; Murakami et al., 2000).

In conclusion, the results in this study suggest that the growth factors (IGF-1 and TGF- β 1), particularly in combination, markedly suppressed cytokine-induced pro-inflammatory degrading enzymes and mediators and enhanced the MAPK signalling pathway and expression of the master chondrogenic transcription factor Sox9, thereby stabilizing chondrocyte differentiation. These intriguing *in vitro* effects of combined growth factors are important observations, potentially adding them to the expanding list of chondroprotective agents that may find clinical application next to other anti-rheumatoid, anti-inflammatory drugs, tissue engineering and ACI.

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