Osteoarthritis and Cartilage (2001) **9,** 382–389

doi:10.1053/joca.2000.0399, available online at http://www.idealibrary.com on IDEAL®

1063–4584/01/040382+08 $35.00/0

Osteoarthritis and Cartilage

Journal of the OsteoArthritis Research Society International

Growth factor expression in cartilage wound healing: temporal and spatial immunolocalization in a rabbit auricular cartilage wound model

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Summary

Objective: The ability of cartilage to regenerate following injury is limited, potentially leading to osteoarthritis. Integrative cartilage repair, necessary for durable restoration of cartilage lesions, can be regarded as a wound healing process. Little is known about the effects of growth factors regulating acute cartilage wound healing *in vivo*. In this study the temporal expression patterns of growth factors and proteoglycan content in cartilage wound edges *in vivo* were studied.

Design: Cartilage wounds were created in rabbit ear cartilage using a 6 mm biopsy punch. Specimens were subsequently harvested 1, 3, 7, 14 and 28 days after surgery. Paraffin sections were thionin stained to visualize proteoglycan loss and replacement. Immunohistochemical staining of TGFβ1, TGFβ3, IGF-1, IGF-II and FGF-2 was used to define growth factor expression at the cartilage wound sites.

Results: Almost no effect of cartilage wounding was observed one day after surgery. A decrease of proteoglycan content, with a maximal loss at day 7, and a subsequent restoration was observed at the wound edges. Growth factor expression increased simultaneously. Maximal immunostaining for IGF1, IGFII, FGF2 and TGF-beta3 was observed at day 7, followed by a gradual decrease. Increased expression of TGFβ1 lasted from day 3 until day 14.

Conclusion: We have demonstrated the ability of chondrocytes to increase growth factor expression and to restore the rapid decrease in proteoglycan content in the initial phase following acute wounding. A temporal increase in intracellular growth factor expression suggests an autocrine and/or paracrine metabolic stimulation, which can be regarded a sign of chondrocytes repair capacity. © 2001 OsteoArthritis Research Society International

Key words: Cartilage, Growth Factors, Immunohistochemistry, Wound Healing, Proteoglycans.

Introduction

Once damaged, hyaline cartilage and elastic cartilage display a poor intrinsic repair capacity, potentially leading to osteoarthritis (OA), airway obstruction or deformation of ears and nose[1–9]. Merely small defects or defects in fetal or very young cartilage show healing[4,5]. Namba *et al*. demonstrated a spontaneous repair *in utero* of partial-thickness defects in articular cartilage in a fetal lamb model[6]. This repair process appears to be absent in matured animals. An important condition for durable repair of cartilage lesions is the integration of wound edges or the integration of repair tissue with the surrounding host cartilage[7]. Failure of cartilage repair caused by an impaired integration has been documented by several authors who studied the natural cartilage repair process[2,8,9]. This failure of repair was also found after transplantation of periosteal and perichondrial grafts[10,11], osteochondral grafts[12], natural[13] or bioengineered grafts[14]. During the transplantation procedure of freshly harvested or bioengineered cartilage the graft and host tissue need to be reshaped in order to achieve a suitable fit into the defect. Thereby fresh wounds are created and the integrative cartilage repair process, necessary for a long-lasting restoration of cartilage, can be regarded as a wound healing process.

Growth factors appear to play an important role in wound healing and regeneration in a number of tissues, e.g. skin[15,16], skeletal muscle[17], gastrointestinal tract[18,19], liver[20] and bone[21,22]. Transforming growth factor β (TGFβ), fibroblast growth factor 2 (FGF-2) and insulin like growth factor (IGF) play a role in various aspects of musculoskeletal tissue regeneration[17,21,23] and remodeling[24].

Despite an increasing number of publications on the effects of growth factors on chondrocyte proliferation and matrix production *in vitro* (for review see[25]) little is known about growth factors regulating cartilage wound healing. Only a few studies report an increase of growth factor expression and decrease of growth factor receptor expression in chronic hyaline cartilage damage, i.e. osteoarthritis[26–28] whereas no studies have documented the spatial and temporal expression of growth factors in acute cartilage wound healing.

Both hyaline cartilage and elastic cartilage are composed of chondrocytes surrounded by an extracellular matrix mainly consisting of collagen type II and aggrecan.

Received 6 December 1999; revision requested 15 February 2000; revision received 31 October 2000; accepted 17 November 2000.

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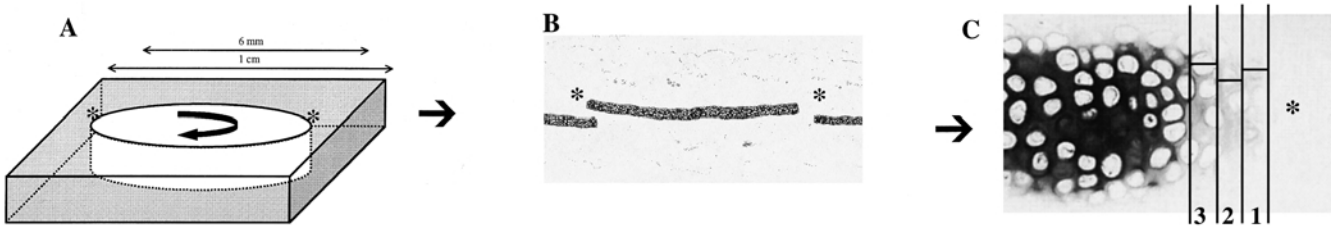

Fig. 1. (A) Schematic drawing of experimental wounding. A 6 mm diameter disc was punched out of the cartilage, rotated and reimplanted. (B) Histologic cross-section of the wounded cartilage. (C) Detail of cartilage lesion edge. The number of cell layers, parallel to the wound edge, with thionin depletion or growth factor expression was scored. In this particular section thionin staining was decreased in three cell-layers. Position of the wound in A, B and C is indicated with asterisks (*).

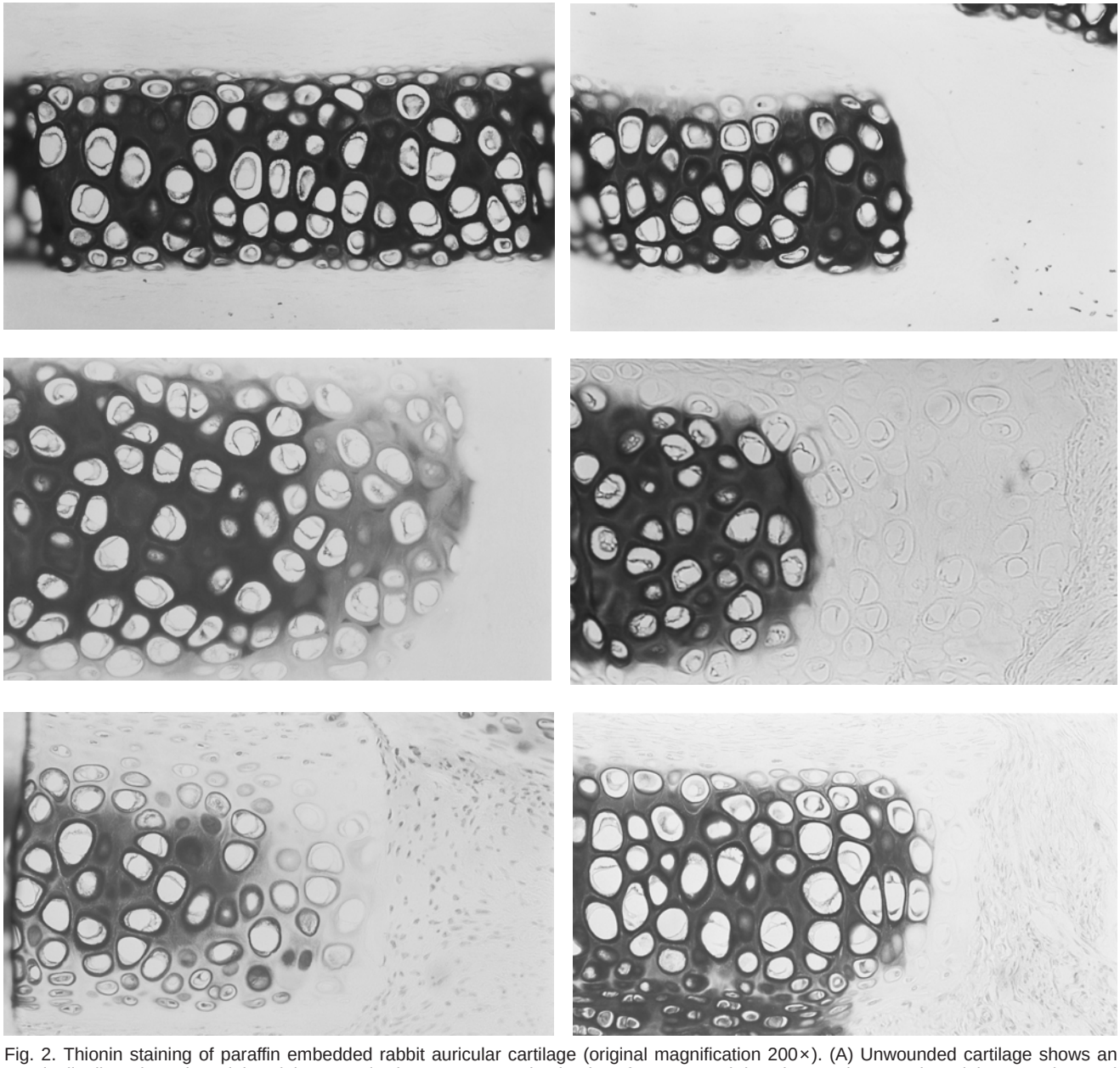

Fig. 2. Thionin staining of paraffin embedded rabbit auricular cartilage (original magnification 200×). (A) Unwounded cartilage shows an evenly distributed purple staining. (B) No matrix changes were noticed 1 day after surgery. (C) At day 3 a decrease in staining was observed at the wound edges, indicating a proteoglycan loss. (D) After 7 days the loss of thionin staining reached its maximum in this sample 7 cell layers. (E) Note the pericellular restorage of staining at day 14. (F) An almost complete restorage of matrix staining was observed at day 28.

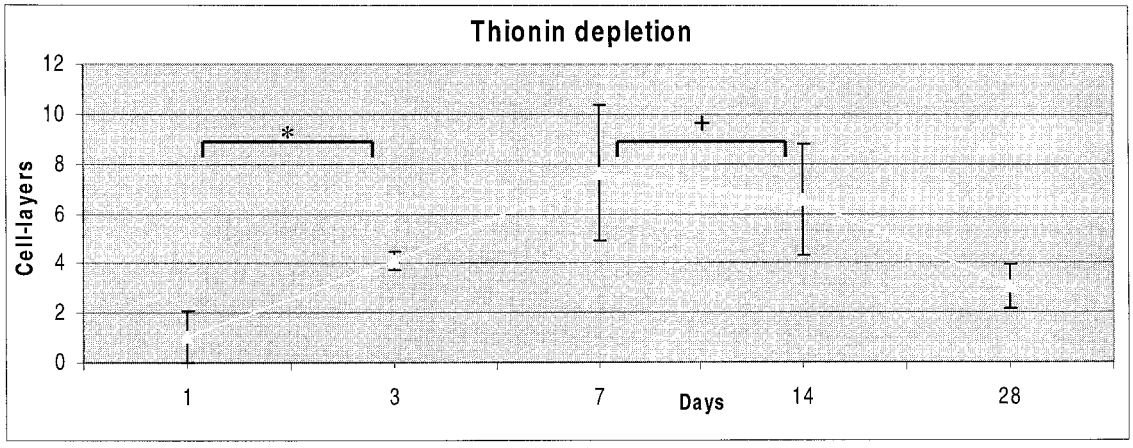

Fig. 3. Number of cell layers partially or completely depleted of thionin staining (Mean±S.E.M. of three rabbits). *: $P<0.05$ +: $P=0.1$.

The chondrocytes of elastic cartilage additionally produce elastin.

In the present study we report temporal and spatial growth factor expression patterns during cartilage wound healing in an auricular cartilage wound-healing model in the rabbit[29]. The immunohistochemical staining of growth factors was demonstrated using antibodies against transforming growth factor β1 and β3, insulin-like growth factors I and II and fibroblast growth factor 2. Expression of growth factors was related to post-injury changes in proteoglycan content in wound areas, as determined by thionin staining[30].

Materials and methods

ANIMAL SURGERY

Nine female New Zealand White rabbits, aged 12–14 weeks (2–3 kg), were used in this experiment. Animal experiments were approved by the Erasmus University Ethics Committee. The animals were housed at the Erasmus Center for Animal Research. The surgical procedures were carried out under semi-sterile conditions.

The animals were anesthetized by an intramuscular injection of 10% ketamine-hydrochloride (Ketalin, Apharma, Arnhem, The Netherlands) 0.5 ml/kg body weight and 2% xylazine-hydrochloride (Rompun, Bayer, Leverkusen, Germany) 0.5 ml/kg body weight. After shaving and disinfecting the skin with 70% ethanol a 2×3 cm angled skin incision was made at the concave (inside) of the ear. The skin was carefully dissected from the perichondrium. An oblique incision, approximately 1cm, was made through perichondrium cartilage and opposite perichondrium and the contralateral skin was bluntly tunneled. Through this cartilage incision a plastic device was introduced between perichondrium and contralateral skin to protect the skin and a 6 mm diameter punch hole was made through cartilage and perichondrium using a biopsy punch (Stiefel, Imported by Bipharma, The Netherlands). The cartilage/perichondrium disks were rotated 180° and reimplanted using three Vicryl 6.0 stitches through the perichondrium (Fig. 1). The skin was closed with Vicryl 6.0 and wounds were dressed with gausses (Bethadine, imported by Asta Medica, The Netherlands) which were removed one day after operation. In three rabbits wounds were created in one ear, the unwounded contralateral ear was used to harvest a control specimen. In the remaining six animals, *two* wounds were created, one wound in each ear.

The animals were killed with an intravenous injection of 1 ml Pentobarbital Natrium 200 mg/ml (Euthesate, Apharmo, Arnhem, the Netherlands). Three cartilage wound samples were harvested immediately after death at days 1, 3, 7, 14 and 28. The circular wounds, including overlying skin, were harvested by dissecting 1×1 cm specimens. Samples were fixed in Carnoy's fixative (ethanol 60%, chloroform 30%, acetic acid 10%) for 20–24 h, processed and embedded in paraffin. Five micrometer sections were cut using a Leica (RM 2135) microtome and placed on poly L-lysine coated slides.

THIONIN STAINING

The sections were deparaffinized in xylene, rehydrated through graded ethanol and stained in 0.04% thionin in 0.01 M aqueous sodium acetate, pH 4.5 for 5 min[30].

IMMUNOHISTOCHEMISTRY

Antibodies directed against growth factors that play a role in musculoskeletal tissue regeneration and remodeling were selected. Not all the members from the TGFβ superfamily were tested; we selected TGFβ1 and TGFβ3 because an opposite effect has been suggested[15]. All steps were performed at room temperature. The sections were deparaffinized in xylene and rehydrated through graded ethanol. After preincubation for 30 min with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 10% normal goat serum to block non-specific antigens, sections were incubated for 2 h at room temperature with antibodies against TGFβ1 (Antihuman TGFβ1, 5 μg/ml, monoclonal mouse IgG, Serotec Ltd, Oxford, U.K.) and TGFβ3 (Antihuman TGFβ3, monoclonal mouse IgG, 5 μg/ml, R&D Systems, Minneapolis, MN, U.S.A.), FGF2 (Antibovine FGF-2, monoclonal mouse IgG, 5 μg/ml, Upstate Biotechnology, Campro Scientific, Veenendaal, The Netherlands), IGF-I (Antihuman IGF-I, mouse monoclonal IgG, 5 μg/ml, Upstate Biotechnology) and IGF-II (Antirat IGF-II, mouse monoclonal IgG, 5 μg/ml, Upstate Biotechnology) diluted in PBS containing 1% BSA and 2% normal goat serum. The antibodies were linked with a

secondary antibody, biotinylated rabbit antimouse immunoglobulins (Super Sensitive Concentrated Detection Kit, Bio Genex, San Ramon, U.S.A.). The secondary antibody was labeled using streptavidine-alkalin phosphatase (Super Sensitive Concentrated Detection Kit, Bio Genex, San Ramon, U.S.A.). Alkaline phosphatase activity was demonstrated by incubation with a New Fuchsine substrate (Chroma, Kongen, Germany), resulting in a red colored signal. In control sections the primary antibody was omitted. For isotype control a mouse monoclonal negative control antibody (mouse IgG1 negative control, Dako A/S) was used. The slides were not counterstained to enable semiquantitative analysis of growth factor expression.

HISTOLOGICAL ANALYSES

The sections were separately scored by two independent observers at a magnification of 200×. In rabbit auricular cartilage chondrocytes are organized in layers perpendicular to the horizontal surface and parallel to the cutting surface [Fig. 1(c)]. Changes in proteoglycan content or growth factor expression can therefore be quantified by counting the number of cell layers. The number of cell layers with decreased or lost thionin staining starting from the wound surface was scored.

In serial sections the cartilage area containing immunopositive chondrocytes was determined by counting (number of positive cell layers) or measuring (distance in μm) starting from the wound edge.

Observed differences in growth factor expression and proteoglycan content were evaluated using Mann–Whitney U-test. Spearman's correlation was calculated for growth factor expression and proteoglycan depletion. The interobserver variabillity was tested using Pearson's correlation coefficient.

Results

All animals recovered well from the operation. All wounds healed well, no signs of infection or perforation of the skin covering the punch wounds were observed. The interobserver reproducibility of scoring cell layers with thionin depletion or growth factor expression was good: the calculated correlation coefficient was 0.885 (two-tailed *P*-value <0.01). The average width of one cell layer calculated for all nine animals was 37.7 (±4.6) μm.

THIONIN STAINING

The extracellular matrix in normal, unwounded auricular cartilage showed a homogeneous thionin staining of the extracellular matrix.

One day after surgery almost no effect of cartilage wounding was observed, i.e. there was minimal reduction in thionin staining. After 3 days, a decrease in thionin staining was observed and after 7 days a maximal thionin depletion with an average of 7.6 cell layers was observed, indicating a considerable loss of proteoglycans in the cartilage wound edges. However, at day 14 there was a recurrence in thionin staining, starting in pericellular areas. After 28 days, an almost complete proteoglycan replenishment was observed (Figs 2 and 3).

IMMUNOHISTOCHEMICAL GROWTH FACTOR EXPRESSION

Growth factor expression in the cartilage was primarily observed intracellularly whereas almost no staining was observed in the extracellular matrix. Omission of the primary antibodies and isotype control, using a mouse IgG1 control antibody confirmed specificity of the immunohistochemical procedure.

Chondrocytes in unwounded, freshly harvested cartilage showed no expression of TGFβ, IGF or FGF-2 [Fig. 4(a)]. One day after surgery minimal growth factor expression could be noticed at the cartilage wound site. After 3 days positive staining for growth factors was found in the chondrocytes of the cartilage wound edges [Figs 4(b) and 5]. The number of cartilage cell layers expressing immunostaining for TGFβ1 and TGFβ3 increased between day 1 and 3, TGFβ1 maintained around that level until day 14. An increase of IGF-I, IGF-II and FGF-2 was observed between days 3 and 7. Staining for IGF-I, IGF-II, FGF-2 and TGFβ3 reached a peak level at day 7 [Figs 4(c) and 5], and showed a gradual decrease afterwards. At day 28 growth factor expression returned to basal levels [Fig. 4(d) and 5].

A small zone of chondrocytes (one to two cell layers) directly bordering the cartilage cutting line did not demonstrate antibody reactivity. From day 7 clear empty lacunae were observed in this zone.

CORRELATION BETWEEN THIONIN DEPLETION AND GROWTH FACTOR EXPRESSION

A simultaneous pattern of growth factor expression and loss of thionin-staining was observed in the cartilage wound area. The area containing positive cell-layers for TGFβ1, IGF-I, IGF-II and FGF-2, as measured from the cartilage wound edges, correlated significantly with the number of cell layers depleted from proteoglycans (Table I).

Discussion

The absence of sufficient natural repair following cartilage injury prohibits durable tissue restoration. In the present study we have shown signs of repair in cartilage exposed to acute wounding. A gradual decrease of proteoglycans at the wound site was followed by a repair reaction demonstrated by a renewed pericellular proteoglycan production. Simultaneously, a temporal increase in growth factor expression was shown in these cells depleted from their normal surrounding matrix. In the final phase, when virtually all lost proteoglycans were restored, growth factor expression was reduced to basal levels. Knowledge on *in vitro* effects of growth factors on matrix production and cell proliferation of chondrocytes and cartilage explants[25] suggest that the temporal expression of endogenous growth factors indicates an autocrine and/or paracrine stimulation of chondrocyte metabolism following acute cartilage injury.

In articular cartilage, the relation between effects of wounding on proteoglycans and chondrocyte metabolism is difficult to study due to the continuous presence of mechanical loading, which influences cell metabolism[31]. Exclusion of mechanical loading by joint immobilization however, will lead to a reduction of proteoglycan content[32]. In this study, we have used ear cartilage to investigate post-injury

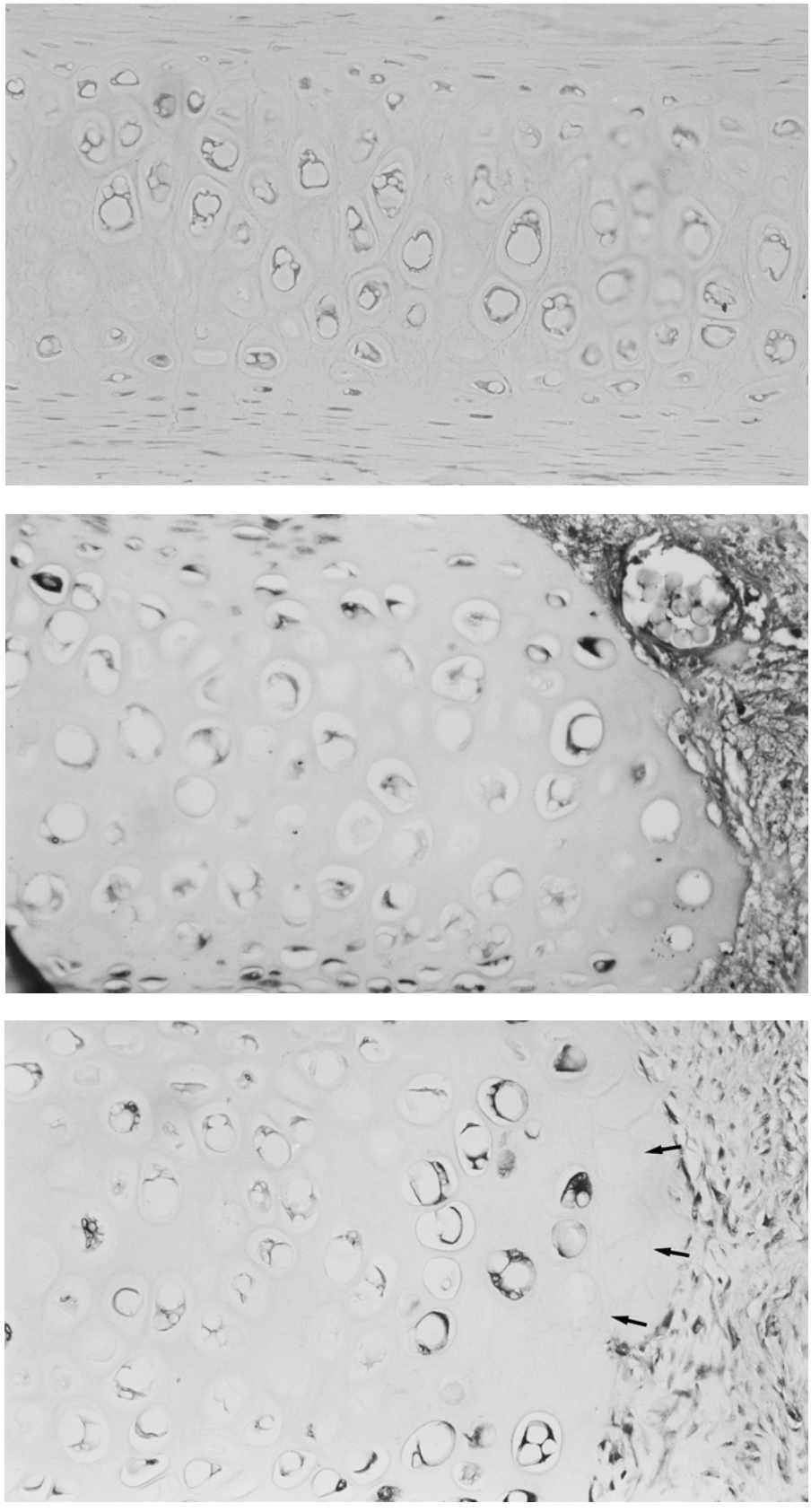

Fig. 4(a)–(c).

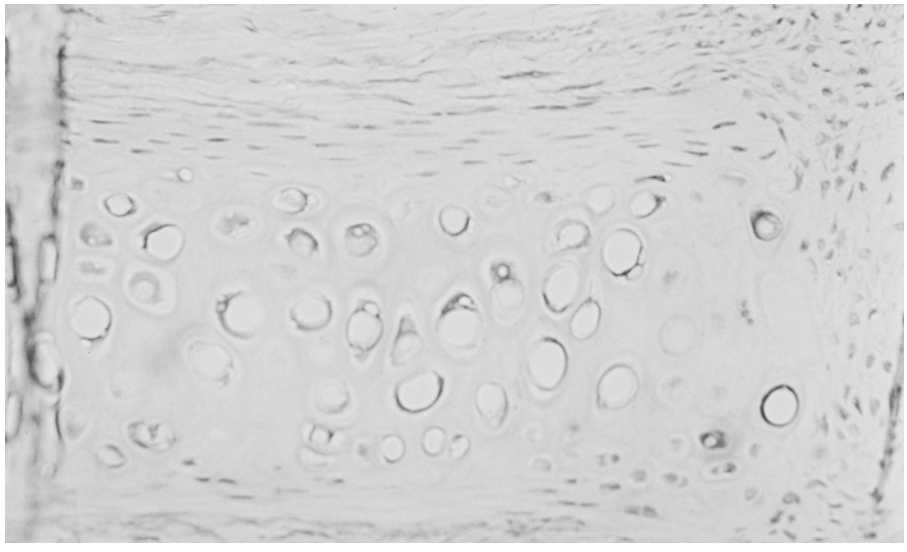

Fig. 4(d).

Fig. 4. Immunohistochemical staining for FGF2 on paraffin embedded rabbit auricular cartilage (original magnification 200×). Note the intracytoplasmatic localization of growth factor signal. (A) In unwounded cartilage no growth factor expression can be observed in chondrocytes. Note that a part of the perichondrium cells show expression of FGF2. (B) At day 3 chondrocytes at the cartilage wound edges start to express FGF2. (C) Maximal expression was noticed 7 days after surgery. Chondrocytes directly bordering the cartilage cutting line do not show expression of FGF2 (arrows). (D) After 28 days the number of cell layers expressing FGF2 was reduced.

changes in proteoglycan content and chondrocyte metabolic response. Ear cartilage is not subjected to the levels of mechanical loading occurring in joints and does not have the limitations of *in vitro* experiments. Additional advantages of our model are the ability to study more than one condition per animal, which requires less extensive surgical procedures and causes less discomfort to the animals.

This is the first report in which the temporal and spatial expression of growth factors, known to be important in musculoskeletal tissue regeneration[17,21,33], were studied in an acute cartilage injury model. A relatively fast increase in TGFβ1 and TGFβ3 expression was observed 3 days after injury. IGF-I, IGF-II and FGF-2 increased to a peak expression at day 7. Previous studies have also demonstrated elevated intracellular expression of growth factors and cytokines in chondrocytes, however these studies were carried out in chronic injured, i.e. osteoarthritic cartilage from patients or animal models[26–28,34]. The experimental setup we have used models the situation encountered following cartilage transplantation.

Currently, much effort is expanded in developing tissue-engineered cartilage transplants to fill up cartilage defects[35]. Successfully developed cartilage transplants, containing the proper amounts and types of collagens and proteoglycans with the appropriate biomechanical properties, still need to integrate into the host cartilage in order to achieve a permanent and durable restoration of the cartilage defect. Integrative cartilage repair is required to enable cartilage transplants to stay in position in the initial (healing) phase, allowing both cartilage wound sites to integrate, and to withstand biomechanical loading patterns in the final phase. For this integration, a wounded edge of the cartilage defect has to form a solid connection with the edges of the transplant.

In spite of the signs of repair we demonstrated after injury, we did not observe integrative repair. Because of the chondrocytes inability to migrate through the matrix, the cells closest to the wound edges are responsible for the integration of both wound sides. However, a part of these cells appeared to die, i.e. first no increased growth factor expression was seen and later empty lacunae were observed. In agreement with our findings other investigators have described cell death following cartilage injury[34]. It is suggested that, like in skin wound healing[36], cells in the wound area of experimentally injured articular cartilage explants die by apoptosis[12]. Recently it has been shown that at least a part of the chondrocytes in osteoarthritic cartilage die by apoptosis[37]. A positive effect in the healing process can be expected from interventions aiming at protecting the viability of chondrocytes aligning the wound site.

In conclusion, we have shown the effects of injury on extracellular matrix and chondrocytes. The suggested relation between growth factor expression and proteoglycan depletion during wound healing and the subsequent replenishment of proteoglycans indicate that cartilage possesses some intrinsic repair capacity. The temporal expression pattern of the growth factors reported here provides the opportunity to study whether adding extra or neutralizing endogenous growth factors can stimulate extracellular matrix production needed for integrative cartilage repair.

Table I
Spearman's correlation coefficient for the area of growth factor expression and the area of thionin depletion

TGFβ1	TGFβ3	IGF-I	IGF-II	FGF2
0.73**	0.44	0.60*	0.69**	0.71**

**P<0.01 *P<0.005.

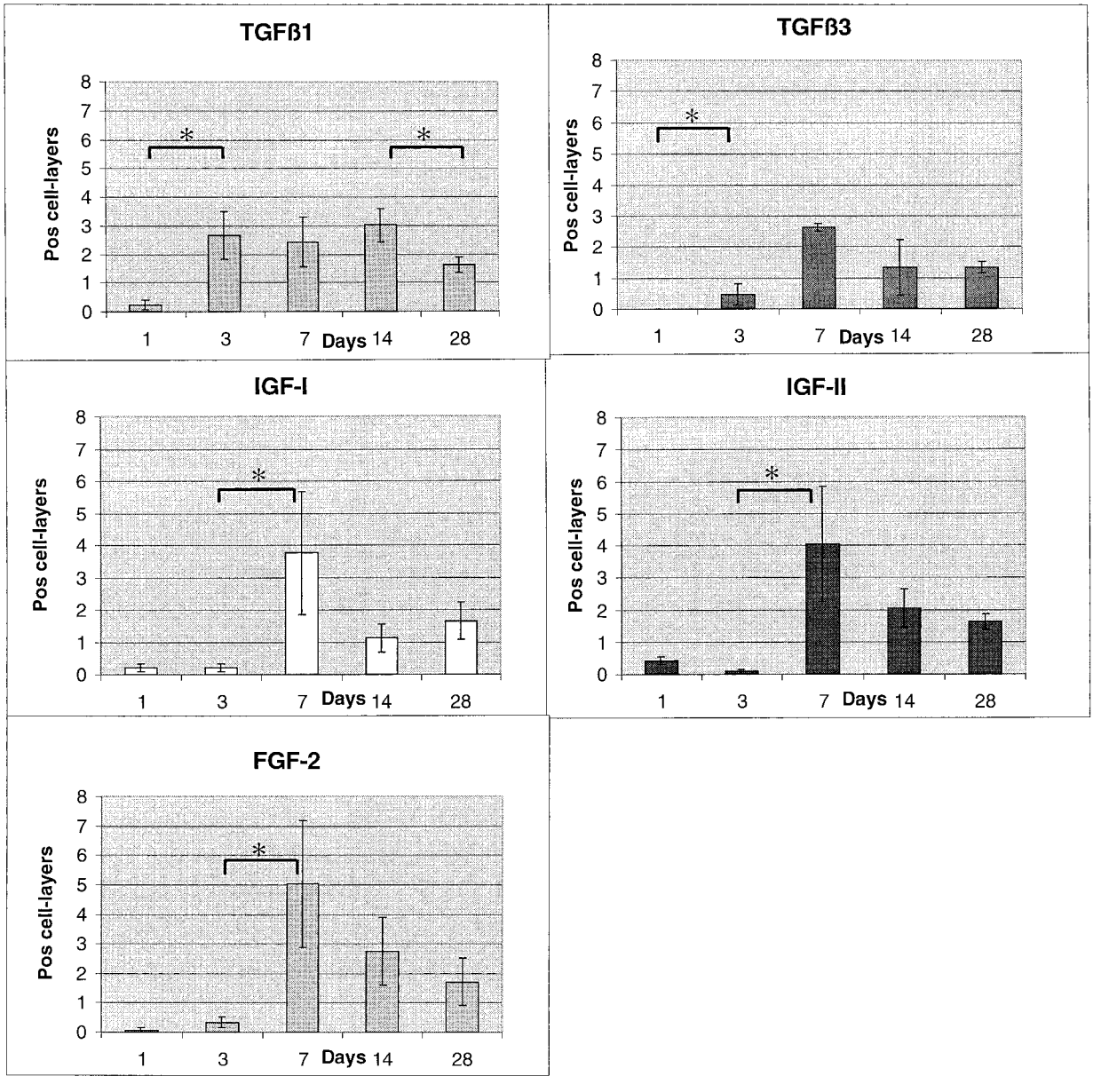

Fig. 5. The number of cell layers positively stained for growth factors (mean±S.E.M. of three rabbits) is represented. A relatively fast increase in expression of TGFβ1 and TGFβ3 was observed between day 1 and 3. Expressions of IGF-I, IGF-II and FGF2 increased between day 3 and 7. Note the clear peak level expression of IGF-I, IGF-II, FGF2 and TGFβ3 at day 7. Peak level expression of TGFβ1 lasted from day 3 to 14. *: $P<0.05$.

Acknowledgments

This project has been supported by the Foundations 'De Drie Lichten' and 'Vereniging Trustfonds Erasmus Universiteit Rotterdam' in The Netherlands. The research of G. van Osch was supported by the technology Foundation STW, applied science division of NWO and technology program of the Ministry of Economic Affairs. The authors would like to thank Dr Paul ten Koppel for development and instruction of the used surgical procedure and Vera Galinovic-Schwartz for technical assistance in immunohistochemical procedures.

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