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Hepatocyte growth factor in human osteoarthritic cartilage

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Summary

Objective: Hepatocyte growth factor/scatter factor is a potent mitogen, morphogen and motogen for a variety of mainly epithelial cells. Hepatocyte growth factor is synthesized by mesenchymal cells and can be found in various tissues. The objective of this study was to investigate the expression and distribution patterns of this pleiotropic growth factor and its receptor, the product of the proto-oncogene *c-met* in normal and osteoarthritic human knee cartilage.

Methods: Five normal and 14 osteoarthritic human cartilage samples graded histomorphologically by Mankin Score, were studied by radioactive in-situ hybridization and immunohistochemistry for the expression of Hepatocyte growth factor and the *c-met* receptor.

Results: Hepatocyte growth factor could be found by immunohistochemistry in the territorial matrix surrounding the chondrocytes of calcified cartilage and within the deep zone of normal cartilage. Chondrocytes of these cartilage zones showed also positive *c-met* receptor-staining. Moreover, a small number of chondrocytes in the superficial and intermediate zone showed *c-met* staining. In accordance with the increased hepatocyte growth factor staining of osteoarthritic cartilage, an enhanced expression of hepatocyte growth factor-RNA by chondrocytes of the deep zone as well as the deeper mid zone was observed. Contrary to normal cartilage, *c-met* was identified immunohistochemically in osteoarthritic chondrocytes of all cartilage zones.

Conclusion: These results indicate that hepatocyte growth factor seems to be acting in an autocrine/paracrine manner in normal and osteoarthritic cartilage. The ubiquitous presence of the HGF/HGF-receptor complex in osteoarthritic chondrocytes suggests that hepatocyte growth factor may contribute to the altered metabolism in osteoarthritic cartilage. © 1999 OsteoArthritis Research Society International

Key words: Osteoarthritis, Hepatocyte Growth Factor, Cartilage repair.

Introduction

Articular cartilage is composed of a highly organized and extensive extracellular matrix (ECM), which constitutes up to 90% of the cartilage weight and a small number of embedded chondrocytes.[1] These chondrocytes synthesize and organize the ECM of the articular cartilage by maintaining a balance between synthesis and degradation of the ECM components. In osteoarthritis (OA), which is known to be the most common cause of pain and disability in the elderly, this balance is disturbed, and a progressive loss of articular cartilage, enhanced chondrocytic cell division and cluster formation are observed.[2] On the other hand, several authors have reported on the potential of cartilage repair mechanisms and described an increased synthesis of extracellular matrix components by OA chondrocytes.[3,4] Furthermore, several cytokines and growth factors could be shown to influence the growth, synthesis and degradation of normal and OA articular joint cartilage.[5–7] Hepatocyte growth factor (HGF) is a heparin-binding glycoprotein which regulates growth and differentiation of many tissues. HGF is expressed by a variety of mesenchymal cells.[8–10] The biological actions of HGF are mediated by the *c-met* proto-oncogene product, a transmembrane tyrosine kinase, which has been identified as the HGF receptor.[11] The dimeric *c-met* receptor has a high affinity-binding region for the heterodimeric polypeptide HGF, which itself is composed of a disulfide-linked 69-kDa α-subunit and a 32 kDa β-subunit.[12] In addition to its interactions with heparin and proteoglycans HGF has been shown to bind various collagens such as types I, III, V and VI.[13,14] Several studies showed that HGF acts in an autocrine/paracrine manner being a key mediator of mesenchymal epithelial interactions.[15,16] HGF is a potent mitogen which induces growth and DNA synthesis in renal tubular epithelial cells, keratinocytes, vascular endothelium and hepatocytes.[17–19] Furthermore, HGF acts as a morphogen inducing renal tubular epithelium in a collagen matrix to form a network of branching tubules.[20] HGF also has motogenic properties for human omental endothelium.[21] A recent study suggests that synovial neovascularization in rheumatoid arthritis can be induced by HGF.[22] In addition, growth of hematopoetic stem cells is stimulated by HGF.[23] HGF and its receptor were also detected in future joint regions of developing limb buds in mouse and rat embryos, suggesting that HGF may have a role in the early articular morphogenesis.[24,25] When HGF was added to chondrocytes *in vitro*, their proteoglycan synthesis and cell-division rate were significantly increased.[25] However, the expression patterns of HGF and its receptor in human normal and osteoarthritic cartilage are largely unknown. Therefore, we used radioactive in-situ hybridization and immunohistochemistry to study the presence and distribution of HGF and its receptor *c-met* in normal and osteoarthritic human joint cartilage.

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Table I
Presence and distribution of HGF protein, HGF RNA and c-met *protein in five normal human articular cartilage samples obtained from five different individuals*

	Superficial zone	Middle zone	Deep zone	Calcified cartilage
HGF protein	−	−	Lower DZ+	+
HGF RNA	−	−	Lower DZ (+) or −	(+) or −
c-met protein	(+)	(+)	Lower DZ+Upper DZ (+)	+

Presence and distribution of HGF protein, HGF RNA compared to *c-met* protein. Relative protein or RNA expression were classified as follows: absent=−; weak=(+), moderate=+, strong=++, very strong=+++ (not present).

Table II
Presence and distribution of HGF protein, HGF RNA and c-met *protein in four mild OA cartilage samples obtained from four different individuals*

	Superficial zone	Middle zone	Deep zone	Calcified cartilage
HGF protein	−	+	++	+
HGF RNA	−	−	+	(+)
c-met protein	(+)	+	++	+

Materials and methods

TISSUE PREPARATION

Fourteen osteoarthritic human cartilage samples were obtained from 14 different patients undergoing total knee replacement. Clinical data were carefully reviewed to exclude any forms of secondary OA and inflammatory joint diseases. Five normal human cartilage samples were obtained from five patients, who underwent amputation. The localizations of the excised samples were carefully documented by video prints. The specimens were immediately fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), decalcified in diethyl-pyrocarbonate (DEPC) treated 0.2 M EDTA, embedded in paraffin wax, and then cut into 6-μm thick sections. Serial sections were stained with safranin-o and hematoxylin/eosin.

HISTOLOGICAL-HISTOCHEMICAL GRADING

Routinely safranin-o stained sections of normal and osteoarthritic cartilage were graded, according to Mankin *et al.*, by three different observers.[26] The cartilage samples were further classified in normal or mild, moderate and severe osteoarthritic cartilage. Mankin score 0: normal cartilage; Mankin score 1 to 4: mild osteoarthritic cartilage; Mankin score 5 to 8: moderate osteoarthritic cartilage; Mankin scores ≥9: severe osteoarthritic cartilage.

Table III
Presence and distribution of HGF protein, HGF RNA and c-met *protein in five moderate OA cartilage samples obtained from five different individuals*

	Upper cartilage	Deep zone	Calcified cartilage
HGF protein	(+)	++	+
HGF RNA	(+)	+	(+)
c-met protein	++	++	+

The remaining upper zones (superficial and middle zones) were summarized in upper cartilage.

IMMUNOHISTOCHEMISTRY

After deparaffinization, the sections were incubated with 2 mg/ml hyaluronidase (Merck, Darmstadt, Germany) in phosphate buffered saline pH 5.5 for 15 minutes, followed by digestion with 1 mg/ml pronase (Boehringer, Mannheim, Germany) in PBS with pH 7.5. Non-specific binding of the antibodies were blocked by incubation with 5% bovine serum albumin in PBS. Each step was followed by extensive washing in PBS. Then sections were incubated overnight at 4°C, with a human HGF specific polyclonal antibody, directed to a 20-mer peptide of the HGF α-chain diluted to 1:20.[14] Adjacent sections were incubated, with a human *c-met* specific polyclonal antibody (Santa Cruz Biotechnology, Inc., U.S.A.), diluted 1:200, and a human type II collagen specific monoclonal antibody (ICN, Meckenheim, Germany), diluted 1:1000. Negative control cartilage sections were incubated with non-immune rabbit serum, respectively. Further, human liver sections served as positive control. After incubation with the first antibody, each step was followed by extensive washing in TBS. Primary antibody were then followed by incubation with a biotinylated anti-rabbit IgG from donkey diluted 1:200, whereas the type II collagen sections were incubated with a biotinylated anti-mouse IgG, diluted 1:500 (Dianova, Hamburg, Germany). Then, a complex of streptavidin and biotin labeled with alkaline phosphatase was added

Table IV
Presence and distribution of HGF protein, HGF RNA and c-met *protein in five severe OA cartilage samples obtained from five different individuals*

	Upper cartilage	Deep zone	Calcified cartilage
HGF protein	+	Lower DZ+	(+)
HGF RNA	(+)	++	(+)
c-met protein	++	++	+

The remaining upper zones (superficial and middle zones) were summarized in upper cartilage. Different results of HGF protein and HGF RNA expression.

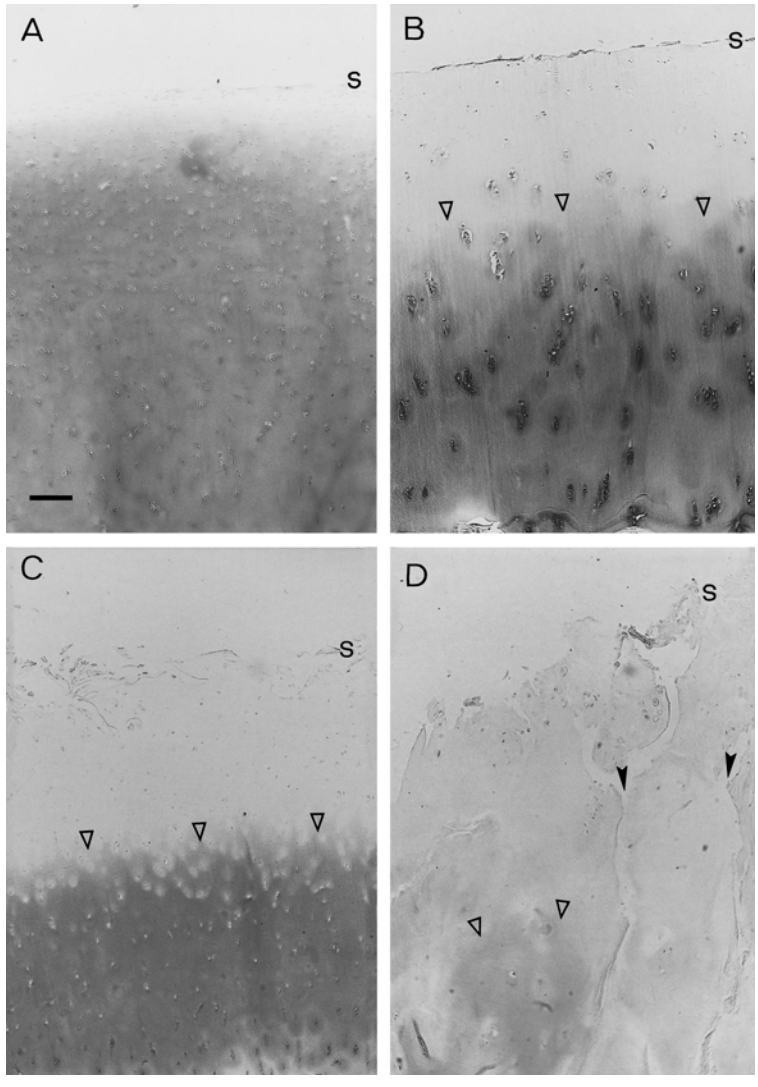

Fig. 1. Safranin-o staining of normal and OA cartilage sections. (A) Normal human cartilage with normal proteoglycan content (s indicates the cartilage surfaces). (B) Mild osteoarthritic cartilage (Mankin-Score 4) with a moderate decrease in proteoglycan content (open arrowheads) and superficial fibrillations. (C) Moderate osteoarthritic cartilage (Mankin-Score 8) with clefts, chondrocyte-clusters and an increased loss of proteoglycans (open arrowheads). (D) Severe osteoarthritic cartilage (Mankin-Score 12) with a diffuse hypocellularity, deep clefts (closed arrowheads) to the calcified cartilage and a great reduction in safranin-o staining (open arrowheads). Bar represents 200 μm in Figs A–D.

according to the protocol of the manufacture (Dako, Hamburg, Germany). Finally the sections were colored with Fast Red (SIGMA, Munich, Germany).

PREPARATION AND LABELING OF PROBES

Probes specific for human HGF and *c-met* were generated by oligo(dT)-primed reverse transcription of human placenta RNA with subsequent amplification oligo-desoxyribonucleotide primers corresponding to nucleotides (sense: 5′-TACTCAAGCTTCCTGGAGTTCCATGATACCACACG-3′), (antisense: 5′-GACTCGGATCCAAGGAATGAGTGGATTTCCCG-3′) of the HGF α-chain, and (sense: 5′-GACCTAAGCTTCACAAAGCAAGCCAGATTCTGC-3′), (antisense: 5′-GACTCGAATTCTGTTGAGTCCATGTCCCGC-3′) of the transmembraneous β-chain of the *c-met* protein. The amplificons were subcloned into PcR3 (Invitrogen, Leek, The Netherlands) and their authenticity was verified by restriction digests and partial sequence analysis. Plasmids were linearized with XbaI or EcoRI restriction endonuclease, to generate [^{35}S]-labeled antisense or sense run-off transcripts (specific activity 1.2–1.4×10^{9} cpm/mg) with T7 or SP6 RNA-polymerase (BRL Gibco, Eggenstein, Germany).

IN-SITU HYBRIDIZATION AND AUTORADIOGRAPHY

Pre-hybridization, hybridization, washing procedures, and RNase digestion of mis-matched sequences as well as autoradiography were performed as described previously.[27,28] In brief, tissue sections were deparaffinized in xylene and rehydrated through graded ethanol, treated with 0.2 M HCl, digested with pronase, fixed in 4% paraformaldehyde/PBS, acetylated, rinsed in PBS, dehydrated in graded ethanols and air-dried. Sections were hybridized at 52°C for 18 hours using 5×10^{5} cpm of

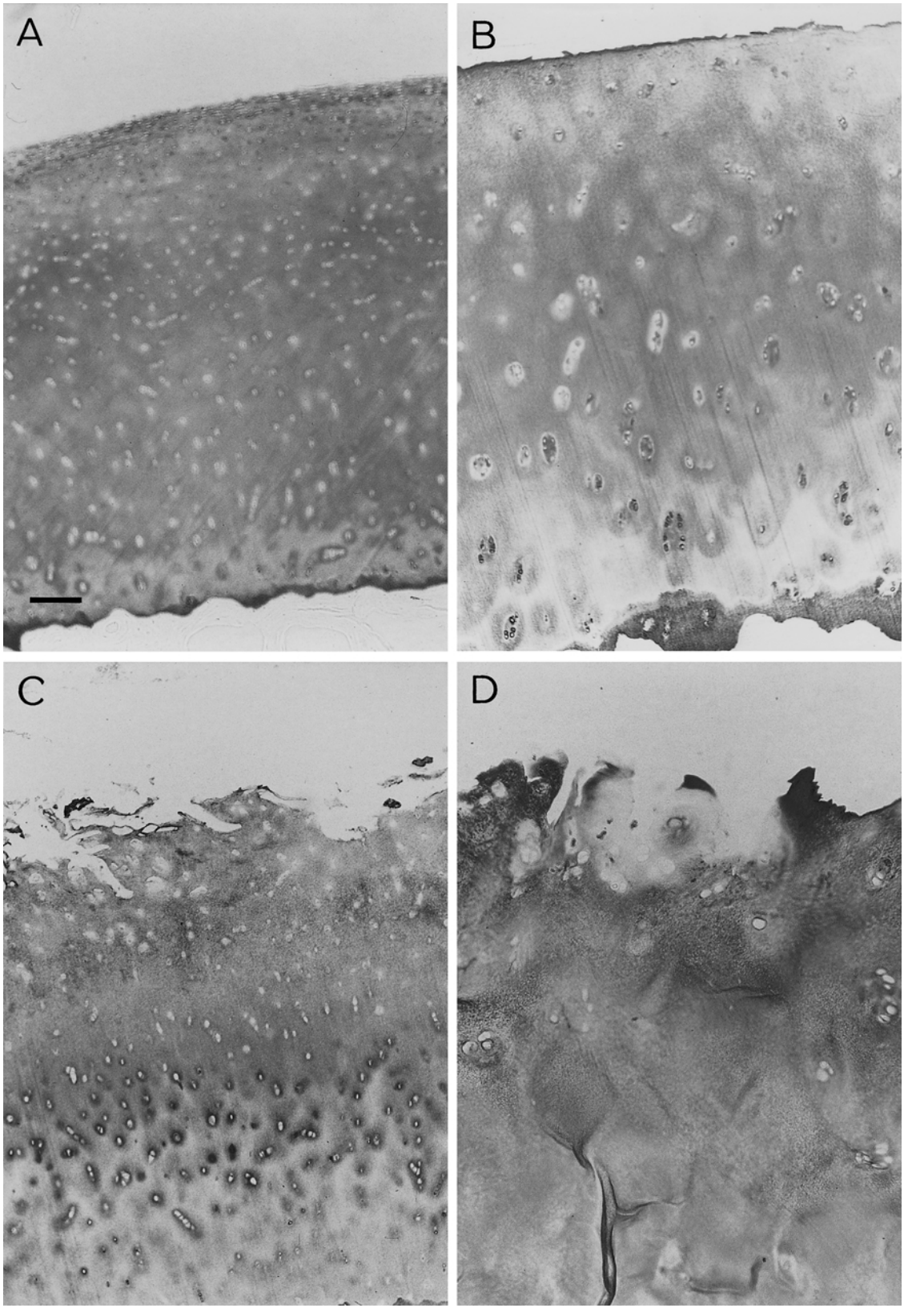

Fig. 2. Immunohistochemistry of type II collagen in normal (A) and OA cartilage sections (B, C and D), using the avidin-biotin-method. Bar represents 200 μm in Figs A–D.

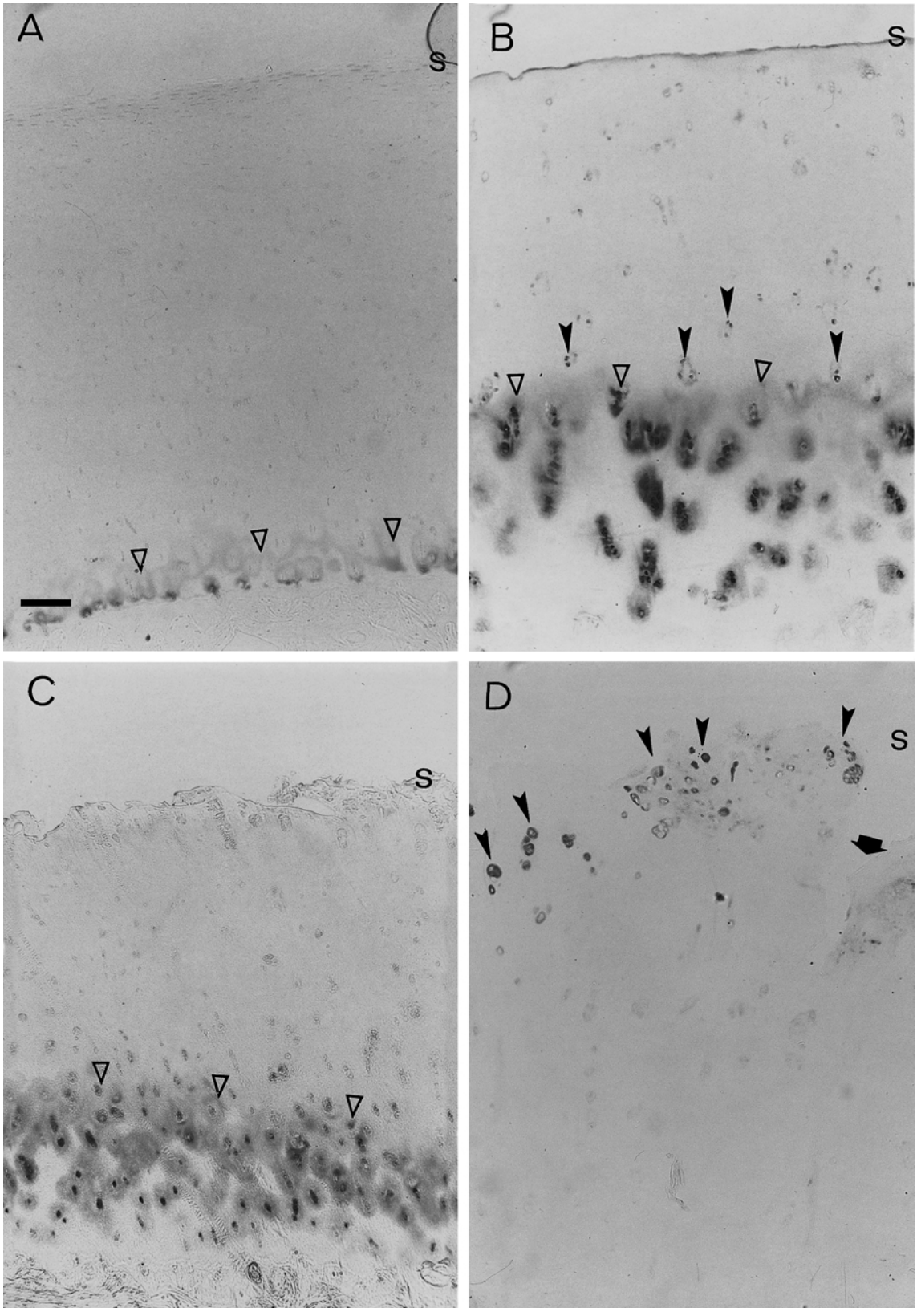

Fig. 3. Immunohistochemistry of HGF in human normal and osteoarthritic cartilage, using the avidin-biotin method. (A) Normal human knee cartilage with a weak staining of chondrocytes within and beneath the calcified cartilage (open arrowheads, s indicate the cartilage surfaces). (B) Mild OA cartilage (Mankin-Score 4) showing an increased number of chondrocytes with positive intracellular and territorial HGF staining in the surrounding matrix (open arrowheads). The chondrocytes beneath show a loss of their territorial HGF staining (closed arrowheads) in close correlation to the loss of proteoglycans (compare the safranin-o staining of the adjacent section Figure 1B). (C) Moderate osteoarthritic cartilage (Mankin-Score 8) with a further increase in the number of cells showing territorial HGF staining (open arrowheads). (D) Severe OA cartilage (Mankin score 12) showing a reduced number of intracellular and pericellular stained chondrocytes (closed arrowheads) in the remaining mid zone next to cartilage surface (arrow indicates a deep cleft). Note that the safranin-o stained adjacent section shows a great reduction of proteoglycans (compare Fig. 1D). Bar represents 200 μm in Figs A–D.

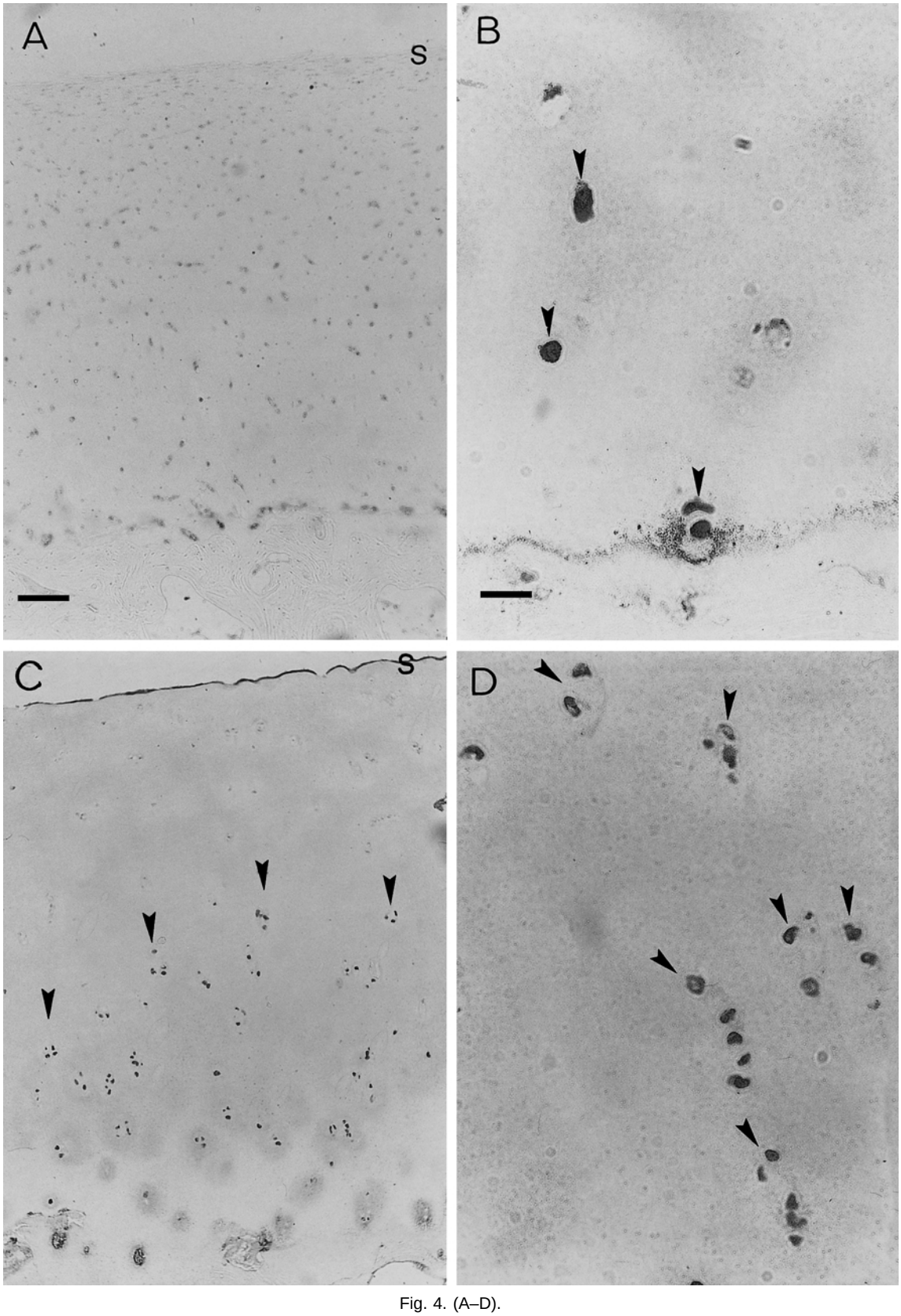

Fig. 4. (A–D).

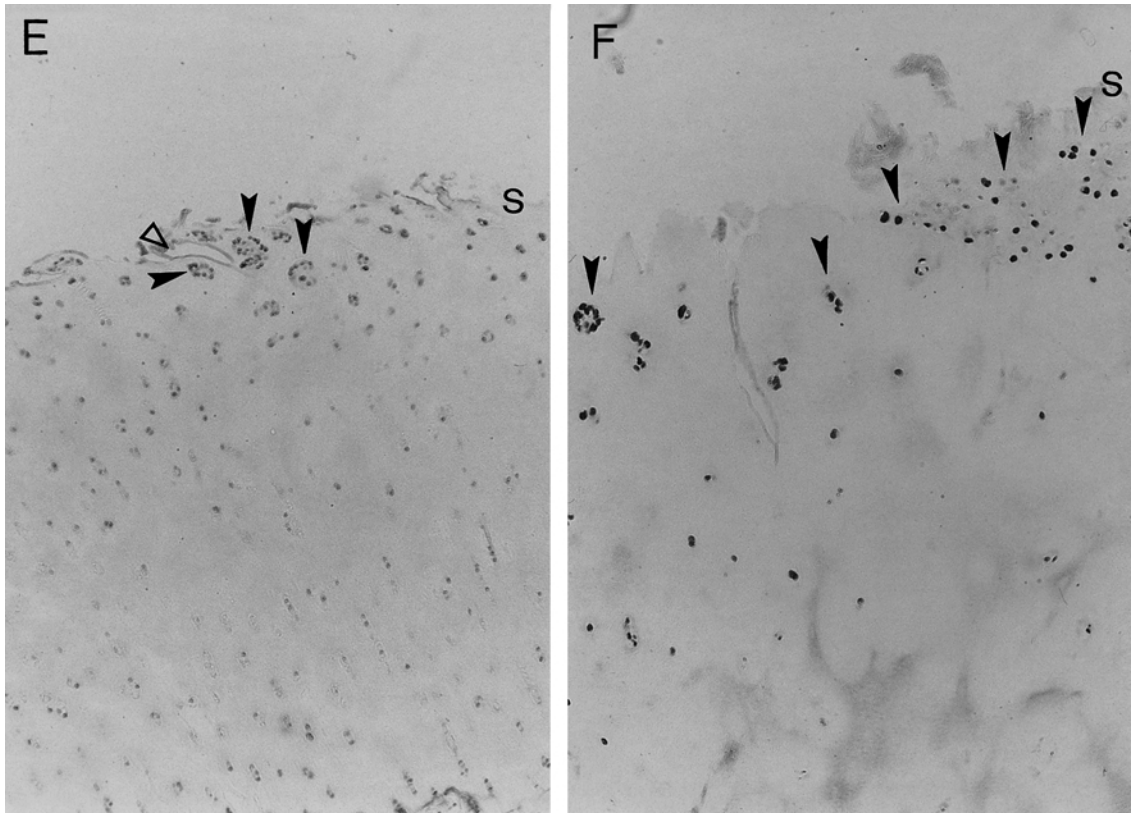

Fig. 4. (E–F).

Fig. 4. Immunohistochemistry for the *c-met* receptor in human normal and osteoarthritic cartilage, using the avidin-biotin-method (s indicates the cartilage surfaces). (A) Normal human cartilage, showing *c-met* positive chondrocytes in the calcified cartilage and in the lower deep zone. In addition several chondrocytes in the middle and superficial zone stain positive. (B) High power magnification of A showing *c-met* stained chondrocytes (closed arrowheads) in the calcified cartilage and the lower deep zone above the calcified cartilage. (C) C-met staining of chondrocytes in mild OA cartilage (Mankin Score 4), showing an increase of *c-met* positive cells (closed arrowheads) in the deep zone compared to normal cartilage. (D) Magnification of C showing the pronounced *c-met* staining of chondrocytes (closed arrowheads) in the deep zone. (E) Moderate OA cartilage (Mankin score 8) showing the majority of OA chondrocytes *c-met* positive. In particular clustered chondrocytes next to a cleft (open arrowhead) show strong *c-met* expression (closed arrowheads). (F) Severe (hypocellular) OA cartilage (Mankin score 12), showing the majority of the remaining chondrocytes positive for the HGF receptor. Bar in A represents 200 μm in Figs A, C, E and F; Bar in B represents 50 μm in Figs B and D.

[^{35}S]-labeled RNA probe, washed for 5 hours at 52°C in modified hybridization buffer, subjected to a brief RNase A digestion, washed briefly, dehydrated in graded ethanols, air-dried, and dipped into Amersham's LM1 nuclear emulsion (Amersham, Braunschweig, Germany). After exposure for 30 days at 4°C, slides were developed in Kodak DI9 developer (Kodak, Hemel Hempstead, U.K.) for 3 minutes, rinsed in 1% acetic acid and fixed in Kodak Fixer for 3 minutes. After extensive washing, the slides were finally counterstained in Hematoxylin-Eosin and mounted in Corbitt balsam. All sections from normal and osteoarthritic cartilage were processed in parallel using the same batches of probes and reagents. Hybridization of tissues pretreated with Micrococcus nuclease verified that cellular RNA was the target of hybridization.

MICROSCOPY

Finally two slides of each immunohistochemical HGF- and *c-met* stained sample as well as one of the sections of anti-sense and sense in-situ hybridizations for HGF and *c-met* were microscopically viewed, by two different observers. The protein and RNA expression was qualitatively graded as follows: absent −, weak (+), moderate + and strong ++ expression of HGF- and *c-met*-protein as well as HGF- and *c-met*-RNA (very strong +++ was not found). These results are summarized in Tables I, II, III and IV.

Results

HGF and its receptor were identified by immunohistochemistry in normal and osteoarthritic human knee cartilage. HGF RNA expression was also detectable by in-situ hybridization, whereas *c-met* RNA could not be found.

In normal human cartilage (Figs 1A and 2A) HGF proteins were found in the territorial matrix surrounding chondrocytes of the lower deep zone and within calcified cartilage (Fig. 3A). Only weak or absent HGF RNA transcripts were identified in these locations by in-situ hybridization (data not shown). Parallel sections incubated with the HGF sense probe (negative control) never showed any signal. The HGF receptor protein was detected in normal cartilage in a cellular or pericellular distribution. HGF receptor staining was observed mainly in the chondrocytes of the

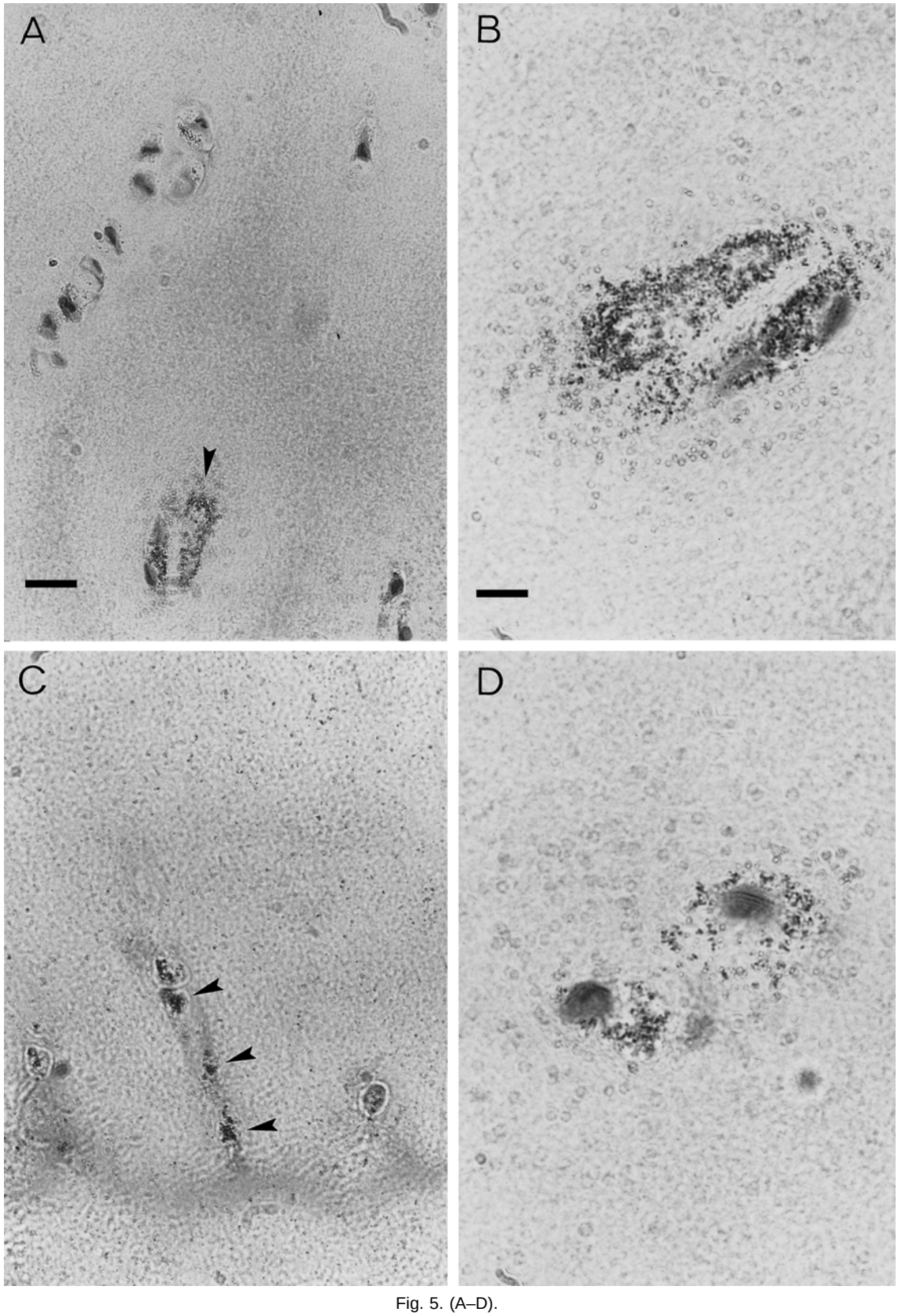

Fig. 5. (A–D).

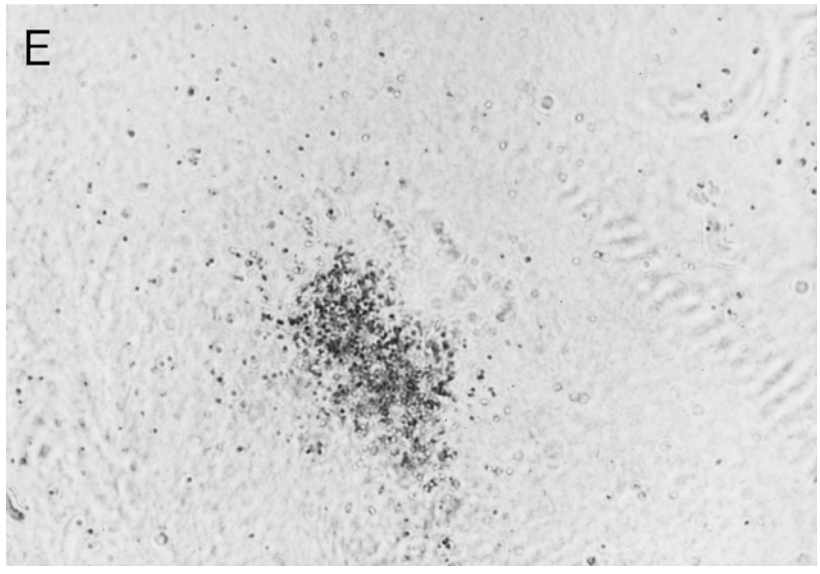

Fig. 5. (E).

Fig. 5. In-situ hybridization for HGF RNA in osteoarthritic cartilage. (A) Mild osteoarthritic cartilage (Mankin score 4) from human knee joints showing HGF RNA with the antisense probe, in the deep zone chondrocytes. (B) Magnification of A showing the HGF RNA Expression. (C) Moderate osteoarthritic cartilage (Mankin score 8) with HGF RNA expression of chondrocytes in the lower deep zone next to the tidemark. Figure (D) and (E) showing exemplary HGF RNA expression of chondrocytes from the deep zone (E) and remaining mid zone (D) in severe OA cartilage (Mankin-Score 10). Bar represents 50 μm in Fig. A and C; Bar in B represents 12.5 μm in Figure B, D and E.

deep zone and within calcified cartilage (Fig. 4A and 4B). Additionally, a small number of mid zonal and a larger number of superficial chondrocytes showed positive *c-met* receptor staining. However, one out of five samples of normal human knee cartilage displayed labeling of *c-met*-protein on chondrocytes of all zones. Immunostaining of this specimen did not show any differences from the above described staining pattern found in the other normal cartilage samples.

Mild OA cartilage (Fig. 1B and 2B) showed an increased number of chondrocytes with an enhanced and enlarged territorial labeling of HGF protein (Fig. 3B). Contrary to the normal control sections, HGF expression was detected in the majority of deep zone chondrocytes (Fig. 5A and 5B). In addition, a few single cells in the fibrillated superficial zone were grained for HGF. In parallel, an overall increase in the number of *c-met*-positive chondrocytes was observed (Fig. 4C and 4D).

In moderate OA cartilage (Figs 1C and 2C) we found a further increase of HGF-stained chondrocytes in the entire deep zone and in the cells directly above the upper deep zone (Fig. 3C). In particular, clustered chondrocytes manifested an enhanced staining intensity. In accordance with the immunohistochemical distribution, the majority of chondrocytes in the mid zone and the deep zone displayed HGF RNA expression as revealed (Fig. 5C). In parallel, the average number of *c-met*-stained chondrocytes was further increased compared to normal cartilage (Fig. 4E).

Severe OA cartilage (Fig. 1D and 2D) characterized by the loss of cartilage tissue, deep clefts within the cartilage, extensive clusters of chondrocytes and a major loss of proteoglycans showed divergent results for immunohistochemically HGF staining and in-situ hybridization of HGF RNA. Territorial labeling for HGF protein was generally decreased, in closed correlation to the great decrease of safranin-o staining (Fig. 3D). Similar to moderate OA cartilage, we found an enhancement of HGF RNA in the deep zone and chondrocytes of the remaining mid zone (Fig. 5D and 5E). As seen in moderate OA cartilage, we found a more pronounced *c-met* staining in most of OA chondrocytes in the remaining cartilage layers (Fig. 4F).

The territorial or interterritorial HGF deposition was positively correlated to the proteoglycan-content of osteoarthritic cartilage. HGF staining in the ECM was only found in regions with almost normal proteoglycan content. A greater loss of proteoglycans in OA cartilage excluded HGF-staining of the ECM. No correlation between the immunohistochemically staining of type II collagen and the HGF deposition could be found.

Discussion

The results of this study demonstrate for the first time that HGF and its receptor, the product of the *c-met* proto-oncogene, are expressed by chondrocytes of adult human articular cartilage as well as chondrocytes of OA cartilage *in vivo*. The increased expression of the HGF/HGF-receptor system in osteoarthritic cartilage, suggest a regulatory role in the homeostasis and pathogenesis of human joint cartilage.

C-met RNA could not be detected by in-situ hybridization, suggesting low constitutional expression levels of the *c-met* RNA in cartilage.

Our observations indicate that in normal human cartilage only a small number of chondrocytes in the calcified and in the deep zone synthesize HGF protein and over these chondrocytes weak or absent HGF RNA expression was detectable suggesting low level expression (see Table I). In the cartilage regions of the deep zone and the calcified cartilage we found moderate HGF receptor staining. However, a few chondrocytes in the superficial as well as in the mid zone also showed positive *c-met* staining. In osteoarthritic cartilage, HGF RNA and protein were increased, this was paralleled by a larger number of *c-met*-stained chondrocytes (see Tables II and III). HGF seems to be

deposited in the territorial matrix of chondrocytes in the presence of proteoglycans, whereas the absence of proteoglycans excluded territorial HGF deposition. Moreover, in severe osteoarthritic cartilage (Mankin score ≥9) we observed a decrease of proteoglycans and territorial HGF protein staining in the presence of enhanced transcript levels for HGF RNA (see Table IV).

HGF was shown to be involved in proliferation and differentiation of many mesenchymal and epithelial tissues. Notably, HGF and *c-met* expression were observed in the somites of mouse embryos, suggesting that HGF may be involved in the development of mesenchymal limb buds.[24] In addition, Takebayashi *et al*. showed the expression of HGF in those areas of limb buds in rat embryos which are going to form joints later on.[25] They also found that chondrocytes *in vitro* respond to HGF with cell proliferation and heightening their total cell number 1.8-fold. In addition, proteoglycan synthesis was also increased 1.5-fold. Additionally, Grumbles *et al*. reported that HGF and *c-met* were expressed in the growth plate of rachitic rats and in chondrocyte cultures of the proliferative zone. They found that HGF increase type II collagen synthesis and alkaline phosphatase activity which is a marker of cell-differentiation.[29] Interestingly, the distribution pattern of HGF shows similarities to the described distribution of type X collagen in OA cartilage which has been interpreted as a molecular marker showing the premature chondrocyte differentiation to hypertrophic cells.[30] Thus it is likely that HGF contributes to the phenotypic shift of OA chondrocytes.

Osteoarthritis is characterized by the enzymatic degradation of the ECM, by various matrix metalloproteinases and an increased synthesis of various cartilaginous matrix components like type II collagen and aggrecan in response to the persistent damage.[4,31–33] This metabolic activation of chondrocytes can be interpreted as a cartilage repair mechanism. In addition, chondrocytes in OA start to proliferate and form clusters. Several cytokines and growth factors have been shown mostly *in vitro* to influence the cellular mechanisms of the osteoarthritic process in cartilage.[7,34,35]

Our observations support the hypothesis that HGF is produced at significant levels in normal cartilage by the chondrocytes of the deep zone, which also show weak to moderate HGF-receptor staining, suggesting an autocrine/paracrine regulatory mechanism of the HGF/HGF receptor system. Growth factors are known to be deposited in the ECM by binding to a variety of matrix components, such as collagens and proteoglycans. In particular, HGF was demonstrated to bind to collagens, thrombospondin, chondroitin sulfate and hyaluronic acid.[14,36] We found a close correlation between HGF deposition in the ECM and the safranin-o staining, suggesting that HGF protein binds to the proteoglycans of articular cartilage. However, HGF attachment to the ECM seems to be independent from type II collagen. Thus, it can be hypothesized that in early OA the degradation of the territorial matrix, as described by Hollander *et al*.,[37] is followed by an increased liberation of HGF, which may induce the synthesis of matrix molecules and cell division. The hypothesis that HGF is involved in cartilage repair is supported by a study of Wakitani *et al*., who observed a complete healing of osteochondral defects in rabbits 6 months after intra-articular injection of HGF.[38]

Moreover, in severe OA cartilage with a major loss of proteoglycans we observed an increased HGF synthesis by in-situ hybridization and the majority of chondrocytes were also positive for the HGF receptor protein. However, the immunohistochemical staining for the HGF protein was only weak or even absent. Only a few chondrocytes of the upper OA cartilage as well as the deep zones showed a pericellular or intracellular HGF staining. Thus, it can be hypothesized that the decreased territorial staining of HGF protein by immunohistochemistry is the result of the loss in proteoglycans which normally retain HGF in cartilage.

In conclusion, we demonstrate that significant amounts of HGF and its receptor *c-met* are expressed in normal and osteoarthritic human joint cartilage *in vivo*. HGF is produced by chondrocytes of the calcified cartilage and deep zone chondrocytes next to the tidemark in normal human cartilage. The HGF receptor *c-met* is present in the calcified cartilage in the deep zone and in a minor fraction of the superficial and mid zone chondrocytes. The synthesis of both HGF and its receptor is up-regulated in OA cartilage. It can be hypothesized that in early OA HGF is deposited in the ECM, but in severe OA its attachment to molecules of the ECM particularly proteoglycans is disrupted and, thus, the biological activity of HGF. On the other hand it can not be excluded that the enhanced expression of HGF/HGF-receptor complex by OA chondrocytes is the result of a general metabolic activation with an increased synthesis in growth factors and cytokines as described recently by Moos *et al*. and other authors.[7,39,40] Future work should clarify the possible physiological functions of HGF in normal cartilage and whether HGF contributes to the cartilage changes and the altered cartilage metabolism associated with OA.

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