Hepatocyte growth factor facilitates cartilage repair

Full thickness articular cartilage defect studied in rabbit knees

Shigeyuki Wakitani[1], Kazuhiko Imoto[1], Tomoatsu Kimura[2], Takahiro Ochi[2], Kunio Matsumoto[3] and Toshikazu Nakamura[3]

Hepatocyte growth factor (HGF) is a multifunctional factor which promotes proliferation, motility and morphogenesis in epithelial cells. In addition, it has been found to play an important role in cartilage metabolism. To investigate articular cartilage repair using HGF in vivo, we injected HGF into rabbit knee joints, where 4 mm diameter osteochondral defects had been made, and observed the animals for 6 months.

We found that HGF effectively repaired osteochondral defects. The repair process of the articular cartilage defects using HGF was shown to be much better than saline injection on all macroscopic and histologic examinations. Although the observation period in our study was short, HGF is one of the most promising candidates for repairing articular cartilage defects clinically.

Departments of Orthopedics, [1]Osaka-Minami National Hospital, 677-2 Kido-cho, Kawachinagano, Osaka 586. Tel 81-721-53-5761. Fax -8904, [2]Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565, [3]Division of Biochemistry, Biomedical Research Center, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565, Japan.
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The capacity of articular cartilage to repair itself is limited (Campbell 1969, Furukawa et al. 1980, Kim et al. 1991). A number of methods to facilitate cartilage repair have been explored. Isolated chondrocyte transplantation seems to be promising (Wakitani et al. 1989) and has been attempted clinically (Brittberg et al. 1994). Transplantation of cells from other tissues, such as periosteal cells, synovial cells and bone marrow cells, is an alternative since these cells are easier to obtain, but the results are poorer than those with chondrocyte transplantation (Wakitani et al. 1994).

Some cytokines/growth factors are known to stimulate chondrocytes to proliferate or to produce cartilage matrix in vitro. These include basic-fibroblast growth factor (bFGF), bone morphogenetic proteins (BMP) and transforming growth factor-ß (TGF-ß). These factors facilitate the repair of articular cartilage defects in animals, but not into a complete hyaline cartilage histologically (Cuevas et al. 1988, Hunziker and Schenk 1994, Seller et al. 1996).

Hepatocyte growth factor (HGF) was first observed to be a protein which promoted the growth of rat hepatocytes in primary culture and was later cloned (Nakamura et al. 1984, 1986, 1989). HGF was also found to be a multifunctional factor that promoted proliferation, motility and morphogenesis in epithelial cells (Matsumoto and Nakamura 1996). In addition to epithelial cells, HGF was reported to play important roles in cartilage metabolism in vitro and in vivo (Myokai et al. 1995, Takebayashi et al. 1995). We investigated the effect of HGF on injured articular cartilage in vivo.

Animals and methods

25 Japanese white rabbits (Nihon Dobutsu Co'LTD, Osaka, Japan) weighing approximately 2.5 kg each were used. All the animals were males, and 6 months old, which meant that their growth plates were closed and that they were skeletally mature, adolescent rabbits. The rabbits were anesthetized by intramuscular injection of a mixture of ketamine (100 mg/mL, 0.60–0.70 mL/kg body weight) and xylazine (20 mg/mL, 0.30 mL/kg body weight). The skin around the knee was shaved anteriorly and washed with 70% ethanol. A parapatellar medial approach was used to expose the knee joint. The patella was dislocated laterally and a 4 mm circular full-thickness defect in the articular cartilage and subchondral bone was made with an electric drill having a 4-mm drill-bit. The hole was placed in the center of the patellar

groove, and its distal edge was placed at the level of the attachment of the extensor digitorum longus tendon. The cone-shaped defect was almost 4 mm in depth and extended through the compact subchondral bone into the cancellous bone in the bone marrow cavity. Then, the wound was closed. The full-thickness defect was made in both knees. All rabbits were returned to their cages after the operation and were allowed to move freely. No animal was observed to have an abnormal gait or impaired locomotion.

In the right knee, 1 μg of human recombinant HGF (Nakamura et al. 1989) dissolved in 0.1 mL saline was injected 3 times a week for 4 weeks (total 12 μg). In the left knee, 0.1 mL saline was injected using the same schedule as a control.

At 4, 6, 8, 12 and 24 weeks after surgery, the rabbits were killed (5 rabbits in each period) and examined macroscopically. The distal part of the femur was fixed in 10% neutral buffered formalin for 1 week, decalcified with 0.5 molar ethylenediaminetetraacetic acid containing 0.1 molar epsilon-amino-n-caproic acid and 0.005 molar benzamidine, and sectioned sagittally, perpendicular to the defect. These sections were obtained from the center of the defect. The specimens were stained with toluidine blue and examined microscopically.

The sections were examined blindly and scored independently by 3 of the authors, without knowledge of the group being examined, using a histological grading scale (Wakitani et al. 1994) composed of 5 categories with a score range from 0 to 14 points (Table 1). Whenever the scores differed, the scorers discussed them and came to an agreement. This scoring was performed again by the same scorers more than 1 week later and the repeatability of the scoring was evaluated.

The difference in scores between the HGF-treated group and the saline-treated group at the various postoperative times was tested using two-way factorial ANOVA, followed by the Mann-Whitney U-test for comparison. The score of the HGF- treated group and that of normal cartilage, which would have a score of zero, were compared at various times and differences were tested using two-way factorial ANOVA. P-values < 0.05 were considered significant.

Table 1. Histologic grading scale for cartilage defect

A. Cell morphology	0	hyaline cartilage
	1	mostly hyaline cartilage
	2	mostly fibrocartilage
	3	mostly noncartilage
	4	noncartilage only
B. Matrix staining (metachromasia)	0	normal (compared to host)
	1	slightly reduced
	2	significantly reduced
	3	no metachromatic stain
C. Surface regularity [a]	0	smooth (>3/4)
	1	moderate (1/2–3/4)
	2	irregular (1/4–1/2)
	3	severely irregular (<1/4)
D. Thickness of cartilage [b]	0	>2/3
	1	1/3–2/3
	2	<1/3
E. Integration of donor to host adjacent cartilage	0	both edges integrated
	1	one edge integrated
	2	both edges not integrated
TOTAL MAXIMUM: A–E	14	

[a] Total smooth area of reparative cartilage compared to the whole area of the cartilage defect.
[b] Average thickness of reparative cartilage compared to that of surrounding cartilage.

Results

Macroscopy

No signs of arthrosis, such as osteophytes, cartilage erosion, or synovial proliferation, were observed in any of the operated knees at any sampling time. This observation is important when we compare the results with those of bFGF or TGF-ß, which induce osteophyte formation.

In the HGF-treated group at 4 weeks after surgery, the distal half of the defect was filled with white opaque tissue, but the proximal half of the defect was filled with shiny, semi-transparent, smooth tissue. The color of the surface was not uniform but was easily distinguishable from the surrounding articular cartilage. At 12 weeks, the reparative tissue became opaque and the color of the surface more uniform, but the margin was still distinguishable. At 24 weeks, the reparative tissue had an appearance similar to that of the surrounding articular cartilage, with the margin of the defect barely discernible (Figure 1).

In the saline control group at 4 weeks, the center of the defect was concave and filled with white shiny tissue, with the margins clearly defined. At 12 weeks, the surface became flat, but its color was not yet uniform. At 24 weeks, although the color of the surface became well incorporated, it appeared irregular (Figure 2).

Histology

In the HGF group at 4 weeks, the defect was almost filled and the predominant tissue looked fibroblastic, with slightly metachromatic staining. However, moderate metachromatic staining was observed in some

Figure 1. Macroscopic appearances of the femoral patellar groove of the HGF injected group 24 weeks after the defect was made. The 4 mm in diameter circle in the center of the femoral patellar groove is the original defect.

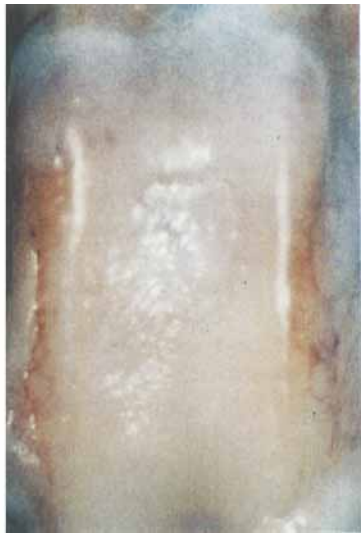
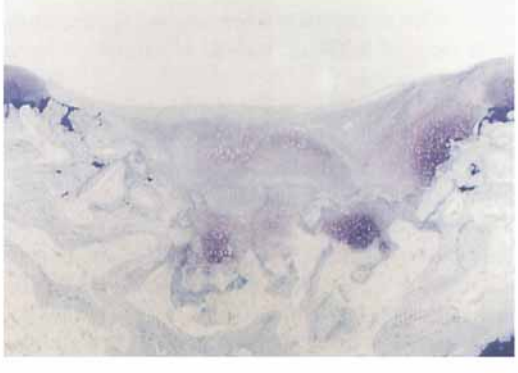


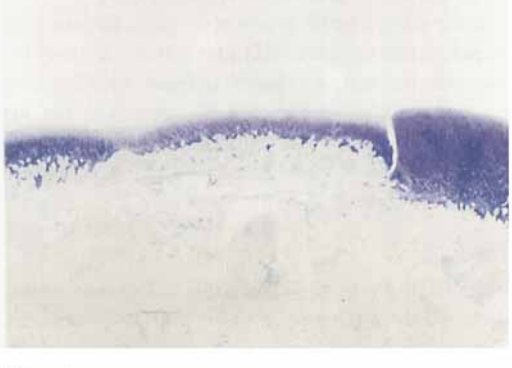
Figure 2. Macroscopic appearances of the femoral patellar groove of the saline injected control group 24 weeks after the defect was made. The 4 mm in diameter circle in the center of the femoral patellar groove is the original defect.

Figure 3. Microscopic appearances of the sagittal section of defect cartilage of the HGF injected group. Toluidine blue staining, x 20.

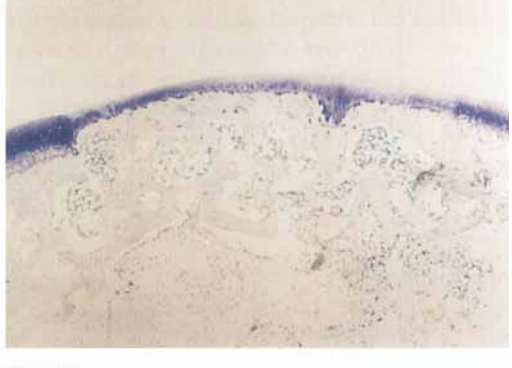


4 weeks.

6 weeks.



12 weeks.



24 weeks.

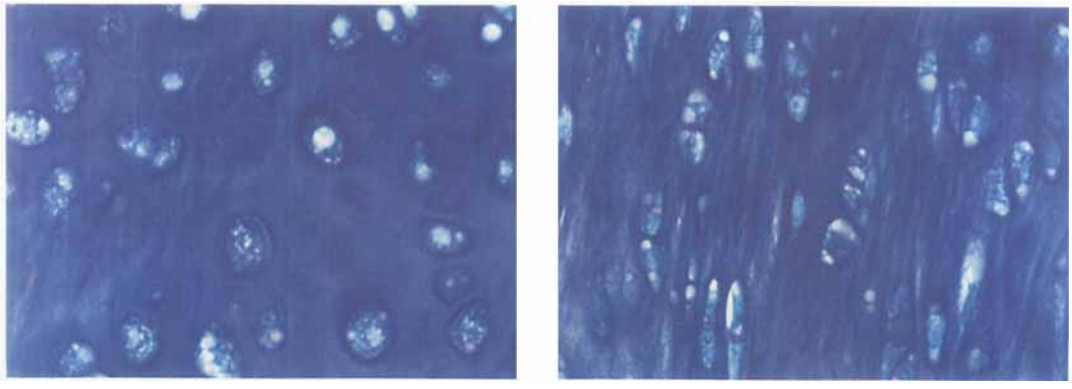


Figure 4. Higher magnification of the sagittal section of defect cartilage of the HGF injected group 6 weeks after the defect was made. Cells in the deep area of the reparative cartilage (left), those in the superficial area (right), Toluidine blue staining, x 200.

areas where cellular morphology consisted of round to polygonal-like articular chondrocytes. At 6 weeks, strong metachromatic staining was observed in almost all areas of the original defect (Figure 3). In the deep area adjacent to the bone marrow, the cells showed a round-to-polygonal morphology, and were surrounded by an intense metachromatic staining, i.e., the cells were hyaline cartilage chondrocytes. In the superficial area of the repaired cartilage, although there existed intercellular matrix with metachromatic staining, the cells were fibroblastic and had no territorial matrix (Figure 4). At 12 weeks, the repair tissue became thinner and was almost the same as the adjacent normal articular cartilage. Metachromatic staining was observed in the whole area of the repaired cartilage, and the cellular morphology of the predominant tissue resembled articular chondrocytes. However, a gap between the repaired cartilage and the host adjacent cartilage was observed in one side, which was seldom observed in the HGF group (2 cases in 25 HGF-treated knees). At the base of the defects, cartilage had been replaced by bone and the subchondral bone was almost repaired (Figure 3C). At 24 weeks, the repaired tissue became thinner than the adjacent normal articular cartilage. Metachromatic staining was still observed in the whole area of the repaired cartilage, although not uniformly. The cellular morphology of the predominant tissue still resembled articular chondrocytes (Figure 3D). At higher magnification, the repaired cartilage 24 weeks after surgery looked rather like the adjacent normal articular cartilage (Figure 5).

In the saline control group at 4 weeks, the original defect was almost filled by fibrous tissue without any metachromatic straining. At 6 weeks, the defects were completely filled, but the reparative tissue had no metachromatic staining. The subchondral bone was partly developed at the bottom of the defect. At 12 weeks, the defects were covered by fibrous tissue containing a little metaplastic cartilage, which was thinner than the adjacent normal cartilage. Subchondral bone was almost repaired. At 24 weeks, the defects were covered by very thin metaplastic cartilage with a faintly metachromatic matrix, the surface was irregular and the cellular morphology of the predominant tissue still looked fibroblastic (Figure 6).

Histology score

In comparison with the saline-treated group, the scores of the HGF-treated group were better using two-way factorial ANOVA ($p < 0.0001$), but significantly worse in comparison with scores for normal cartilage ($p < 0.0001$).

The scores of the HGF group were significantly better than those of the saline group at each postoperative time-point, except for that 4 weeks after surgery, using the Mann-Whitney U-test (Table 2).

Although the repaired cartilage (category D) became thinner with a longer observation time, the other 4 categories became better at 6 weeks and were stable until 24 weeks after the defect was made.

Discussion

It is generally accepted that immature articular cartilage has a greater repair capacity than mature cartilage, and that the capacity declines with age (Buckwalter et al. 1987). Thus, articular cartilage of adolescent rabbits is thought to have a repair capacity intermediate between immature and adult ones. If a growth factor has enough effect on cartilage repair, it would be preferred clinically because these factors are much easier to use than is tissue transplantation. Although the observation period in our study was

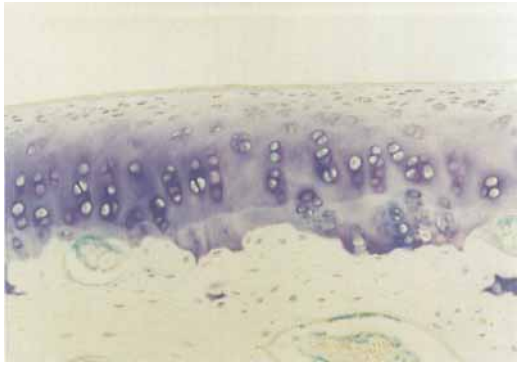


Figure 5. Higher magnification of the sagittal section of defect cartilage of the HGF injected group 24 weeks after the defect was made. Toluidine blue staining, x 100.

Table 2. Result of histologic grading scale

Weeks after surgery	histologic grading scale A	B	C	D	E	total Mean	SD	P-value [a]
HGF injected								
4	2.0	1.8	1.8	1.4	1.6	8.6	1.7	0.3
6	1.6	0.6	1.4	0.0	1.0	4.6	1.7	0.01
8	1.6	1.0	0.8	0.0	1.0	4.4	1.9	0.04
12	1.8	1.2	0.4	0.4	0.8	4.6	1.9	0.03
24	1.6	1.0	0.4	1.2	0.8	5.0	1.8	0.03
saline injected								
4	2.6	2.2	1.6	1.8	1.6	9.8	1.4	
6	2.4	2.0	1.6	1.6	1.4	9.0	1.4	
8	2.0	2.0	1.6	1.4	1.0	8.0	2.3	
12	1.8	1.6	1.4	1.4	1.2	7.4	1.1	
24	2.4	1.8	1.2	1.4	1.4	8.2	1.6	

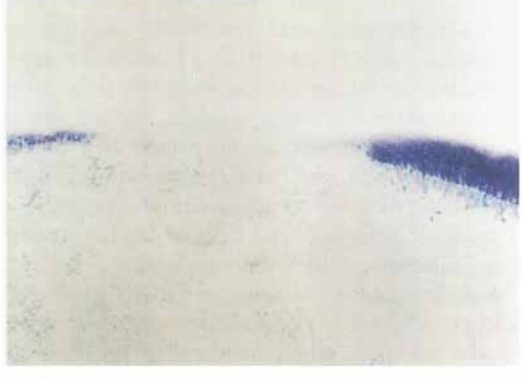
[a] compared with that of saline group at corresponding time.

short and although there remain problems to be solved, such as the thinning of reparative cartilage, HGF was shown to have a remarkable effect in repairing articular cartilage defects. HGF may be a promising candidate for clinical repair of articular cartilage defects. It has no apparent species specificity (Matsumoto and Nakamura 1992).

In the natural repair of articular cartilage defects,

Figure 6. Microscopic appearances of the sagittal section of defect cartilage of the saline injected control group, x 20.

4 weeks.



6 weeks.

12 weeks.

24 weeks.

chondrocyte progenitor cells are thought to migrate into the defect from adjacent bone marrow, then proliferate and differentiate into chondrocytes (Shapiro et al. 1993). HGF is thought to be effective during some stage of this chondrogenesis. In our study, although we injected HGF or saline for 4 weeks and gave no further treatment afterwards, major differences between the groups devloped after 6 weeks. This suggests that the progenitor cells of chondrocytes were stimulated to migrate into the chondrogenic pathway before 4 weeks and that, once this migration was initiated, the cells kept migrating into the pathway. At 6 weeks, the reparative cartilage occupied the whole area of the original defect and was much thicker than the adjacent normal articular cartilage. However, after that time, chondrocytes below the subchondral bone line were replaced by bone and the thickness of the reparative cartilage became thinner than normal articular cartilage.

The effect of HGF on proliferation of cultured rabbit chondrocytes reaches a maximum at 3 ng/mL, and remains almost the same at 10 ng/mL (Takebayashi et al. 1995). The HGF-effect on DNA synthesis in rat hepatocytes in primary culture reaches a maximum at 5 to 8 ng/mL, and the activity remains almost constant at higher concentrations up to as much as 100 ng/mL (Matsumoto and Nakamura 1992). The concentration of HGF in our study was 10 μg/mL, which is 10^2–10^3 times higher. We have no data on the in vitro effect of such high concentrations of HGF, but we observed no ill effects of this. We found that when we injected 100 μL of dye, it spread all over the knee joint (data not shown). Injection of 100 μL of 10 μg/mL HGF is enough to obtain an effective concentration at the defect site.

Although we have no data on the half-life of HGF in the rabbit knee joint, it is presumed to be short because the half-life of TGF-ß in the rat knee joint has been reported to be less than 1 hour (Fava et al. 1991). Because both TGF-ß and HGF are peptides, their half-lives are supposed to differ only slightly. Continuous injection using an osmotic pump may be more effective.

Shida et al. (1994) and van Beuningen et al. (1994) reported that a single injection of bFGF or TGF-ß into joints stimulates articular cartilage and subsequently osteophyte formation. They found that chondrocytes in the articular cartilage responded, but immature cells, such as periosteal cells in the edge of the femoral condyle, responded much more and the thickened cartilage became bone. It is possible that some cytokines stimulate arthrotic changes in joints. In our study, we found no cartilage thickening, no synovial proliferation and no osteophyte formation. HGF seems to have no effect on normal chondrocytes, periosteal cells or synovial cells, and it has been reported to have mitogenic effects on chondrocytes, but not on other mesenchymal cells (Takebayashi et al. 1995).

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