Biochemical and Biophysical Research Communications **261,** 131–138 (1999)
Article ID bbrc.1999.0991, available online at http://www.idealibrary.com on IDEAL®

TAK-778, a Novel Synthetic 3-Benzothiepin Derivative, Promotes Chondrogenesis *in Vitro* and *in Vivo*

Haruhiko Akiyama,*[,1,2] Akira Fukumoto,*[,1] Chohei Shigeno,† Hiromu Ito,* Shogo Mukai,* Tetsuo Hoshino,‡ Haruhiko Makino,‡ and Takashi Nakamura*

**Department of Orthopaedic Surgery, †Calcium Laboratory, Department of Nuclear Medicine and Diagnostic Imaging, Graduate School of Medicine, Kyoto University, Sakyo, Kyoto 606-8507, Japan; and ‡Pharmaceutical Research Division, Takeda Chemical Industries, Ltd., Osaka, Japan*

Received June 11, 1999

TAK-778, a novel synthetic 3-benzothiepin derivative, stimulates the formation of cartilaginous nodules in mouse chondroprogenitor-like ATDC5 cells *in vitro* in association with upregulation of the gene expression of transforming growth factor-β_2, but not bone morphogenetic protein-4 and insulin-like growth factor-I. One-shot injection of the TAK-778-containing sustained-release microcapsules accelerated the repair process of the full thickness defects of articular cartilage in rabbit knees. Our *in vitro* and *in vivo* results indicate that TAK-778 may be a therapeutically useful synthetic agent for articular cartilage repair.

Many patients with arthrosis may benefit from successful joint resurfacing. However, articular cartilage is notorious for the limited capability to repair itself. Previous attempts toward joint resurfacing have gained some favorable results as well as drawbacks: Injection into the joint cavity of growth/differentiation factors such as fibroblast growth factor (FGF)-2 and bone morphogenetic protein (BMP)-2 resulted only in histological improvement of the regenerated articular surface [1, 2] while these affected the surrounding normal articular cartilage and lead to the chondrophyte formation. Transplantation of autologous chondrocytes has recently been applied clinically [3]. Although good results have been obtained at least in a short-term period, surgical intervention is prerequisite to this method. We thought that the synthesis of a new agent, which selectively acts on cartilage lesions without an effect on normal articular cartilage upon local administration, will prove useful as a therapeutic agent for joint resurfacing.

We previously reported that ipriflavone (7-isopropoxyisoflavone) enhanced bone-like tissue formation *in vitro* due to stimulation of differentiation of rat bone marrow stromal cells [4]. This finding led us to search for compounds with more potent effects on cellular differentiation of mesenchymal cells, especially chondroprogenitors. In this study, we evaluated the effects of TAK-778, a novel 3-benzothiepin derivative [5], on chondrogenesis during a process of the articular cartilage repair *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials. In a search for therapeutic agents, we found that 2-benzothiopyran-1-carboxamide derivatives, derived from ipriflavone as a lead compound, increased cellular alkaline phosphatase activity in cultures of rat bone marrow stromal cells. Further modification has led to the more potent 3-benzothiepin-2-carboxamide derivatives. Of these, TAK-778, (2*R*,4*S*)-(−)-*N*-[4-(Diethoxyphosphorylmethyl)phenyl]-1,2,4,5-tetrahydro-4-methyl-7,8-methylenedioxy-5-oxo-3-benzothiepin-2-carboxamide, was selected for further investigation as a new drug stimulating chondrogenesis [5].

Cells and culture conditions. ATDC5 cells were cultured as previously described [6]. In the present study we plated ATDC5 cells in a log growth phase in 24- or 6-multiwell plastic plates (Corning, New York, NY, U.S.A.) at an initial cell density of 4×10^4 or 8×10^4 cells/well respectively and cultured these cells with DMEM/Ham's F12 hybrid medium (Flow Laboratories, Irvine, U.K.) containing 5% (vol/vol) FBS (GIBCO BRL, Gaithersburg, MD), 10 μg/ml human transferrin (Boehringer Mannheim GmbH, Mannheim, Germany), 3×10^{-8} M sodium selenite (Sigma Chemical Co., St. Louis, MO) and 10 μg/ml bovine insulin (Wako Pure Chemical, Osaka, Japan) (the differentiation medium) in a 5% CO_2/95% air atmosphere. Under these conditions, undifferentiated ATDC5 cells reached confluence on day 5, cellular condensation occurred on day 7 and cartilaginous nodule formation occurred on day 9 [6].

Measurement of cartilage-specific proteoglycan. ATDC5 cells were plated in 24-multiwell plastic plates and cultured in the differentiation medium. After 2 days, the medium was replaced with fresh medium containing TAK-778 (10^{-7}, 10^{-6} and 10^{-5} M, each) or vehicle and the culture was continued for further 10 days with medium

[1] The first two authors equally contributed to the work.

[2] To whom correspondence should be addressed at Department of Orthopaedic Surgery, Graduate School of Medicine, Kyoto University, Sakyo, Kyoto 606-8507, Japan. Fax: 81 75 751 8409. E-mail: akiy@kuhp.kyoto-u.ac.jp.

0006-291X/99 $30.00

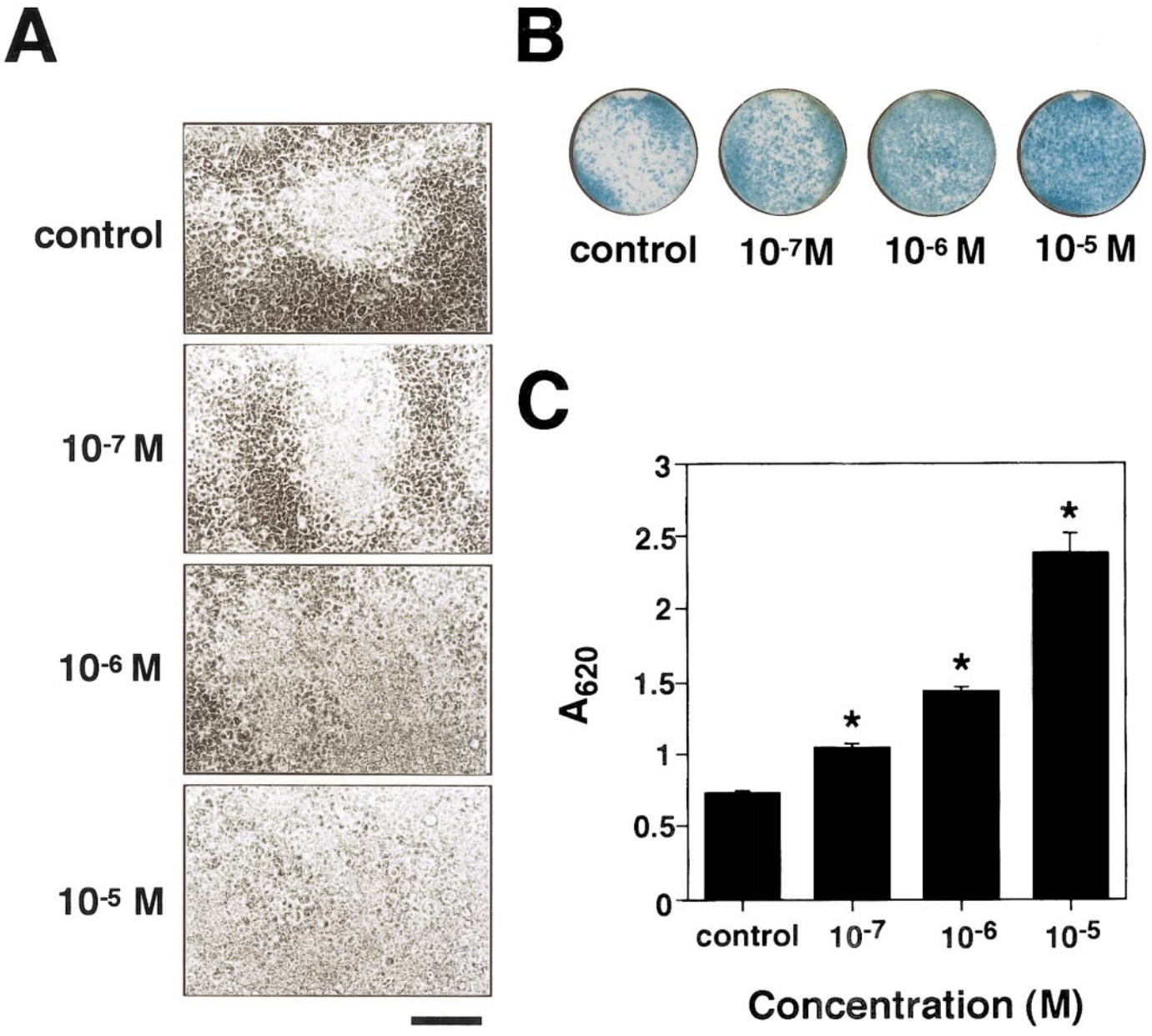

FIG. 1. Effects of TAK-778 on the cartilaginous nodule formation, and Alcian Blue-stainable proteoglycan content. Undifferentiated ATDC5 cells were plated as described under Materials and Methods and cultured for a total of 12 days with TAK-778 (10^{-7}, 10^{-6} and 10^{-5} M, each) and its vehicle (day 2–12). Cells were then assessed for cartilaginous nodule formation under a phase-contrast microscope (A), or stained with Alcian Blue (B). Alcian Blue-stainable proteoglycan content (C) was determined. Original magnification, ×100. Bar denotes 100 μm. Data are the mean ± SD of five wells, and are representative of three similar experiments. *Statistically significant difference from the corresponding control groups at $p < 0.001$.

change every other day. Cartilaginous nodule formation was assessed under a phase-contrast microscope on days 12. After a total of 12 day incubation, cells were rinsed twice with ice-cold PBS (−), fixed with methanol (2 minutes, −20°C), rinsed once with distilled water, and then stained overnight at room temperature with 0.1% Alcian Blue (Alcian Blue 8 GX, Sigma Chemical Co., St. Louis, MO) in 0.1 N HCl. Cells were rinsed three times with distilled water, and the amount of cell-associated dye was measured at 620 nm after extraction with 6 M guanidine-HCl (400 μl/well) [7]. Statistical significance was assessed by one-way analysis of variance and unpaired Student's t-test.

RNA extraction and northern analysis. ATDC5 cells were plated in 6-multiwell plastic plates and cultured for the indicated time periods and cells were exposed to the different doses of TAK-778 or their vehicle. Total RNA (20 μg) was denatured, separated by 1% agarose gel electrophoresis, and transferred on Nytran membranes (Schleicher & Schuell, Dassel, Germany) [6, 7]. The following cDNA fragments were used as hybridization probes: a 0.33 kb cDNA fragment for TGF-β_2 cDNA, and a 0.6 kb cDNA fragment for BMP-4 cDNA. After hybridization, the membranes were exposed at −80°C to X-Omat films (Kodak, Rochester, NY) with Cronex lightening plus intensifying screens (DuPont, Boston, MA).

TABLE 1

Scores on the Histological Grading Scale for Cartilage Defect

	Category*					
	Cell morphology	Matrix staining	Surface regularity	Thickness of cartilage	Integration of donor to host adjacent cartilage	Total score†
TAK-778 (n = 6)	1.5	1.2	0.5	0.5	0.0	3.7**
Control (n = 6)	2.7	2.5	1.8	0.3	0.0	7.3

† Values are given as the average.

* Values are given as the average score for each category, as assessed by two different investigators.

** The total scores for the TAK-778-treated group were significantly different ($p < 0.05$) from those for the control group.

RT-PCR. The RT-PCR was performed as described previously [6]. Briefly, first-strand cDNA was synthesized using SuperScript II RNase H- reverse transcriptase (GIBCO BRL, Gaithersburg, MD) with total RNA extracted from ATDC5 cells. The oligonucleotide primers for PCR amplification of *IGF-I* as follows; 5′-GGACCAGAGACCCTTTGCGGGG-3′ (sense primer) and 5′-GGCTGCTTTTGTAGGCTTCAGTGG-3′ (antisense primer). The PCR conditions were 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds for 32 cycles, and final extension at 72°C for 5 minutes. The primers were specific for mouse glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene (Clontech, Palo Alto, CA) used as a positive control for cDNA amplification. Aliquots (8 μl) of each PCR products were resolved on 3% NuSieve 3:1 agarose gels (FMC Bioproducts, Rockland, Maine) alongside the markers. The amplified products were stained with ethidium bromide. The amplified PCR products were subcloned into pCRII (Invitrogen, San Diego, CA), and the nucleotide sequences of all cDNA fragments were verified by an ALF Express Sequencer (Amersham-Pharmacia, Uppsala, Sweden).

Preparation of TAK-778-containing sustained-release microcapsules. In this study we used TAK-778-containing sustained-release microcapsules consisted of a biodegradable polymer, copoly (Dl-lactic/glycolic acid) (PLGA). PLGA with a copolymer ratio 85/15 mol/mol and an average molecular weight 15,000 (Wako, Osaka, Japan) was used. A mixture of TAK-778 and the PLGA (1:9 w/w) were dissolved in dichloromethane. This solution was poured into an aqueous 0.1% polyvinyl alcohol solution with stirring by a homogenizer to make an oil in water emulsion. To evaporate the dichloromethane, the oil in water emulsion was further stirred for 3 hours. After removing large particles by sieving, the resulting microcapsules (30 μm in diameter) were collected by centrifugation and then lyophilized into a powder. When the microcapsules suspended in 0.5 ml of a dispersing vehicle containing 2.5% D-sorbitol, 0.9% NaCl, 0.1% polysorbate 80, 0.5% calboxymethylcellulose sodium, and 0.07% Na_2HPO_4 were subcutaneously injected into rats, the microcapsules released TAK-778 over four weeks after the injection (data not shown).

Osteochondral defect models. Twelve male Japanese white rabbits (Japan SRL Co., Hamamatsu, Japan) weighing 3.0–3.5 kg were anesthetized with an intravenous injection of pentobarbital sodium (30 mg/kg of body weight) and an intramuscular injection of xylazine (1.6 mg/kg of body weight). Knee joints were approached through medial parapatellar incision. Single full-thickness osteochondral defect, 3 mm wide and 3 mm deep, was drilled through the articular cartilage into the subchondral bone in the trochlear groove of each femur using a hand drill. All rabbits were returned to their cages and allowed to move freely immediately after recovery from anesthesia. Twelve days after operation, animals were anesthetized with an intramuscular injection of ketamine (40 mg/kg of body weight) and xylazine (1.6 mg/kg of body weight), and TAK-778 (5 mg)-containing sustained-release microcapsules (60 mg total) suspensed in 250 μl of medium was injected into the knee joint on one side in each animal. The knee joint on the other side in each animal received injection of 60 mg of sustained-release microcapsules only and served as a control. Under the conditions of a control, a repair process of the full thickness defects of articular cartilage is completed at twelve weeks after operation (data not shown).

Histological evaluation. The rabbits were sacrificed at 4 weeks after TAK-778 injection. The distal part of the femurs was fixed in 10% neutral buffered formalin, decalcified in Gooding and Stewart solution, and embedded in paraffin. Four mm thick serial sagittal sections were stained with hematoxylin-eosin, toluidine blue or safranin O-fast green, and subjected to histological grading of cartilage repair tissues as previously described by Wakitani *et al.* [8]. Statistical significance was assessed by the Mann–Whitney *U*-test.

RESULTS

Effects of TAK-778 on chondrogenesis in ATDC5 cells. During a repair process of the full thickness defects of articular cartilage, marrow-derived mesenchymal cells may exhibit the repertoire of reparative cells including chondroprogenitors. Recently, we and others showed that clonal mouse EC cells, ATDC5, display a number of characteristics as committed to chondroprogenitors [6, 7, 9, 10]. When cultured in the presence of insulin, ATDC5 cells form cartilaginous nodules (chondrogenesis), and enter into sequential chondrocytic maturation processes. In the present study, therefore, we assessed the effects of TAK-778 on chondrogenesis in chondroprogenitors using ATDC5 cells.

In undifferentiated ATDC5 cells, TAK-778 markedly stimulated in a dose-dependent manner the formation of cartilaginous nodules and the accumulation of Alcian Blue-stainable cartilage-specific proteoglycan (Fig. 1A–C). We recently found that undifferentiated ATDC5 cells express BMP-4, TGF-β_2 and IGF-I, which then play roles in regulating chondrogenesis in these cells (data not shown). TAK-778 upregulated in time- and dose-dependent manners the gene expression of TGF-β_2 but not BMP-4 or IGF-I in undifferentiated ATDC5 cells (Fig. 2A–D). Both the recent evidence that TGF-β_2 is a potent promoter of chondrogenesis both *in vitro* [11] and *in vivo* [12] and our *in vitro* results support the notion that TAK-778, during the process of the articular cartilage repair, may stimulate

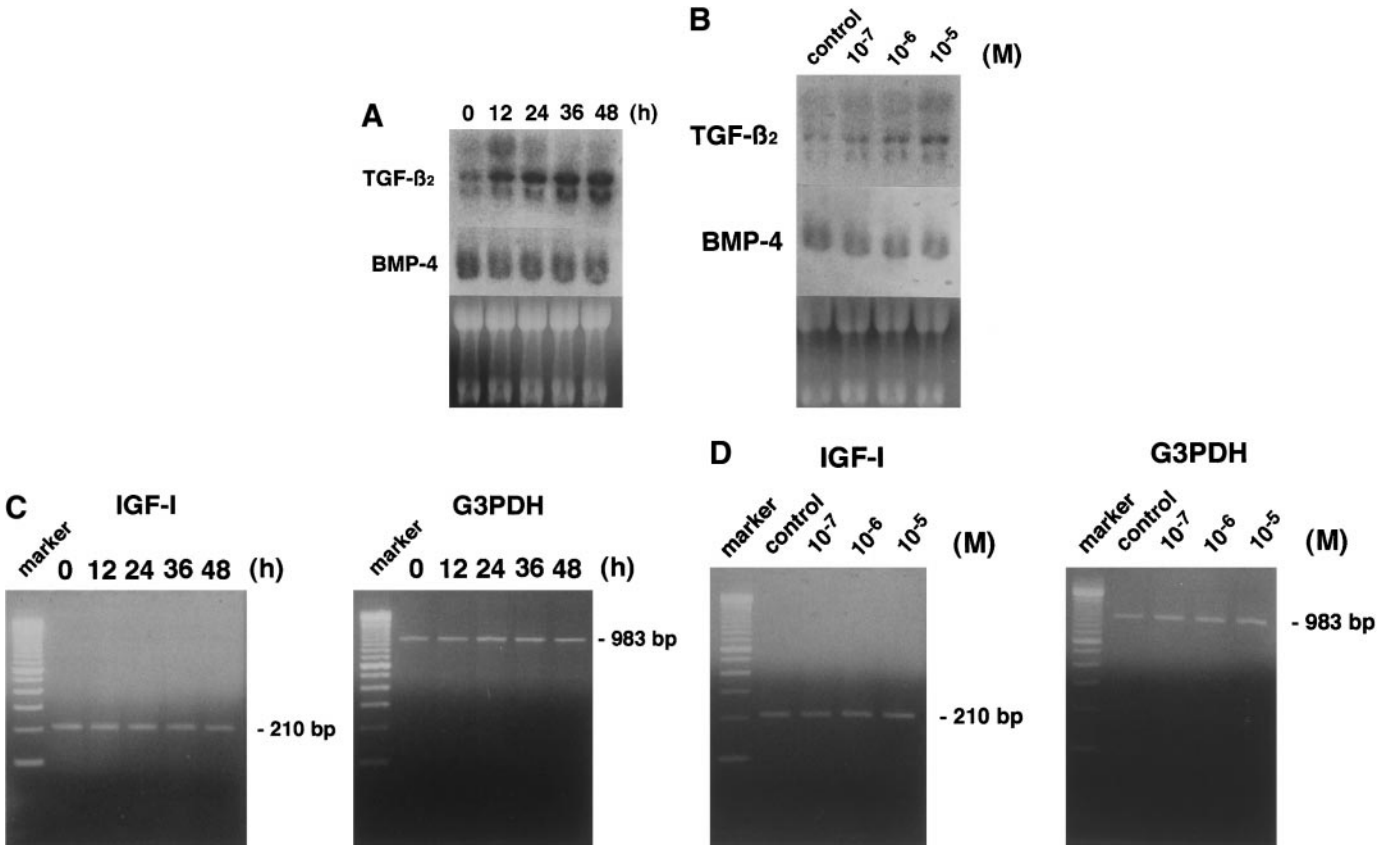

FIG. 2. Time- (A, C) and dose- (B, D) dependent modulation of the transcript levels of TGF-β_2, BMP-4 and IGF-I genes in undifferentiated ATDC5 cells. Cells were grown in the differentiation medium for 5 days, and then exposed either for the indicated time periods to TAK-778 (10^{-5} M) or for 48 hours to either vehicle or TAK-778 (10^{-7}, 10^{-6} and 10^{-5} M, each). Total RNA was prepared as described under the Methods section and subjected for Northern analysis (20 μg of total RNA per lane) (A, B) or RT-PCR (C, D). The lower panel shows ethidium bromide staining of the gel. Three independent experiments were performed and gave similar results.

TGF-β_2 expression and thereby promote chondrogenesis in chondroprogenitors.

Results of TAK-778 in a rabbit model of full thickness osteochondral defects. We used a rabbit model of full thickness osteochondral defects in knee joints and administered TAK-778 as sustained-release microcapsules by one-shot injection into the joint cavity. We used adult Japanese white rabbits (3.0–3.5 kg) and made a single defect, 3 mm wide by 3 mm deep, in the trochlear groove and histologically evaluated the articular cartilage repair 4 weeks after injection. In the control group, the defect was filled entirely with fibrous tissue: Its extracellular matrix did not stain either with safranin O-fast green or metachromatically with toluidine blue, thus lacking the staining characteristics pertinent to cartilage (Fig. 3a–c). On the other hand, in the TAK-778 treated group, the defect was completely filled with cartilage-like tissue stained with safranin O-fast green as well as metachromatically with toluidine blue (Fig. 3d–f). Histologically, the repair tissue appeared indistinguishable from the surrounding normal articular cartilage and subchondral bone except the discontinuities between the repair and normal tissues. The articular surface of the repair cartilage was smooth, and near complete restoration up to the original bone-articular junction was observed. Moreover, the cells in the repair tissue were round or polygonal in shape, morphologically similar to the articular chondrocytes (Fig. 3g and h). Furthermore, the chondrophyte formation was not observed (Fig. 3i). When evaluated by the histological grading scale for cartilage defect by Wakitani *et al.* [8], TAK-778 treated defects displayed significantly better scores than controls (treated vs control; 3.7 ± 0.5, 7.3 ± 0.6, mean $\pm$ SD ($n = 6$), $p < 0.05$) (Table 1).

DISCUSSION

Chondrogenesis is initiated by transient cellular condensation of mesenchymal chondroprogenitors to form a cartilaginous core where chondrogenic differentiation takes place to yield differentiated chondrocytes. We recently showed that chondrogenesis of ATDC5 cells initiates and promotes endogenously expressed

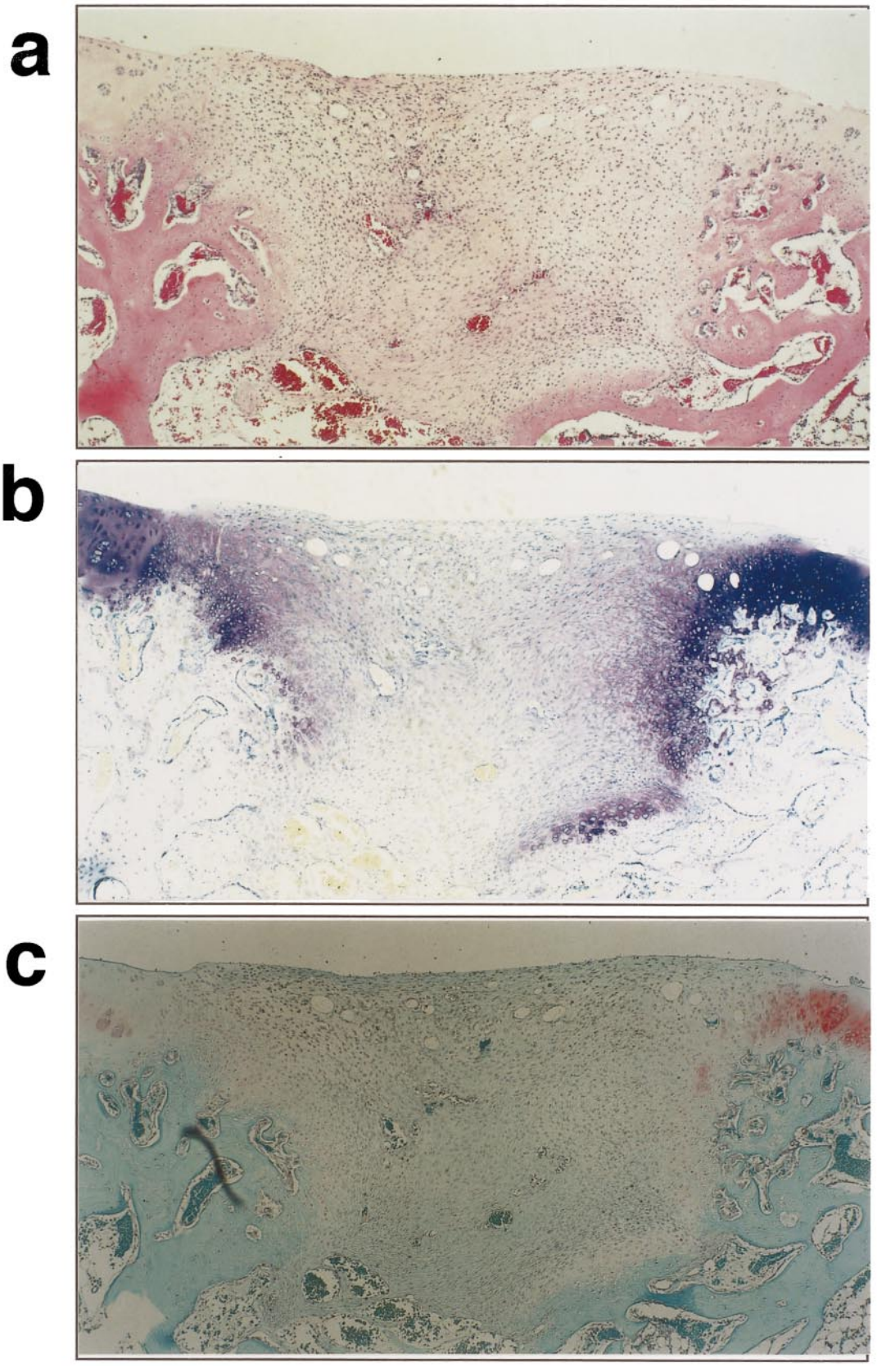

FIG. 3. Histological appearance of the repair tissue in full-thickness osteochondral defects of rabbit knee joints at 40 days after operation. The knee joints received injection of sustained-release microcapsules only served as a control (a–c, g) and TAK-778-containing sustained-release microcapsules were injected into the knee joints (d-f, h, i): (a, d) hematoxylin-eosin staining, (b, e) toluidine blue staining and (c, f) safranin O-fast green staining of the repair tissue were shown. Original magnification, ×40. (g, h) Reparative cells were observed by toluidine blue staining. Original magnification, ×200. (i) The surrounding normal cartilage was shown by hematoxylin-eosin staining. The arrow indicated the repair tissue. Original magnification, ×40.

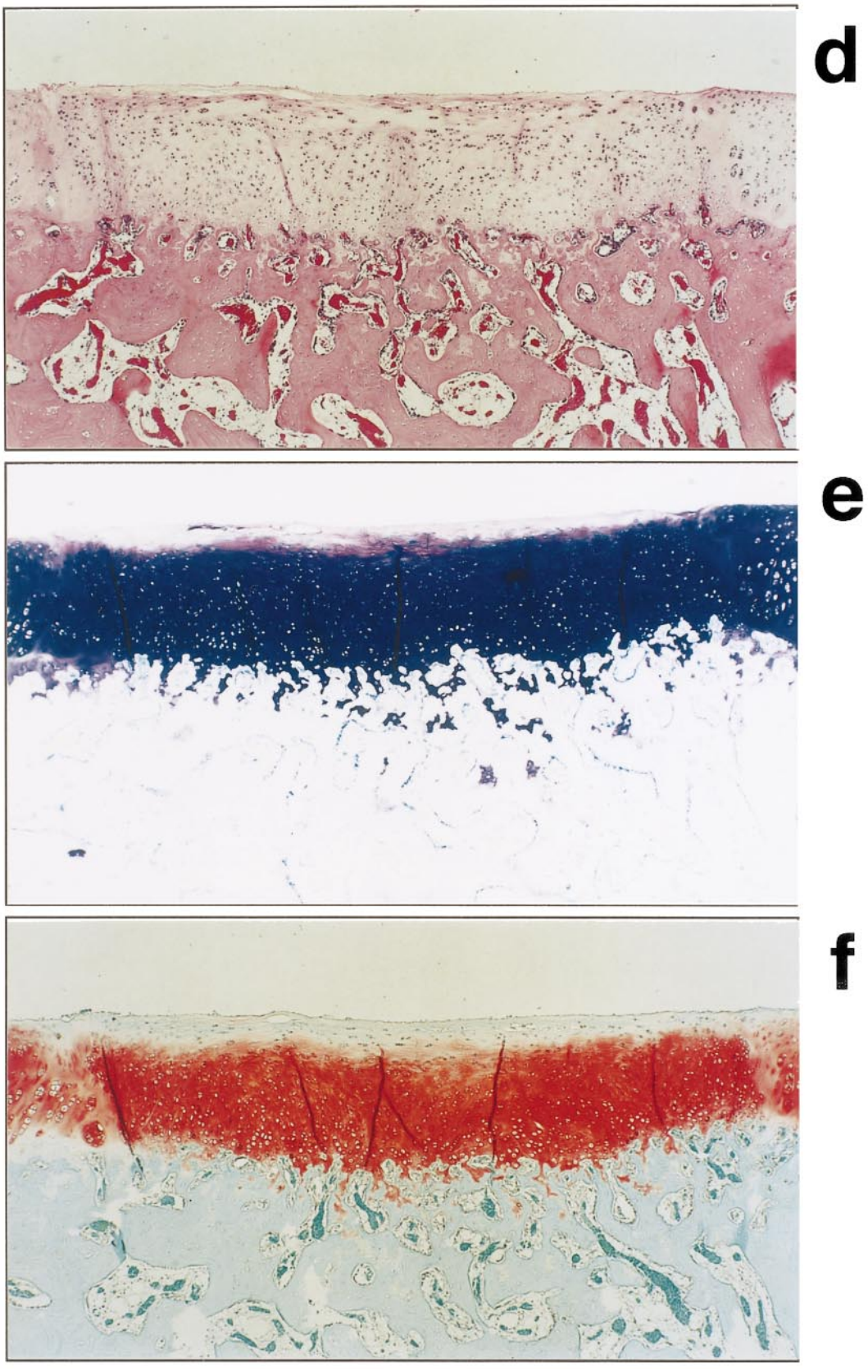

FIG. 3—*Continued*

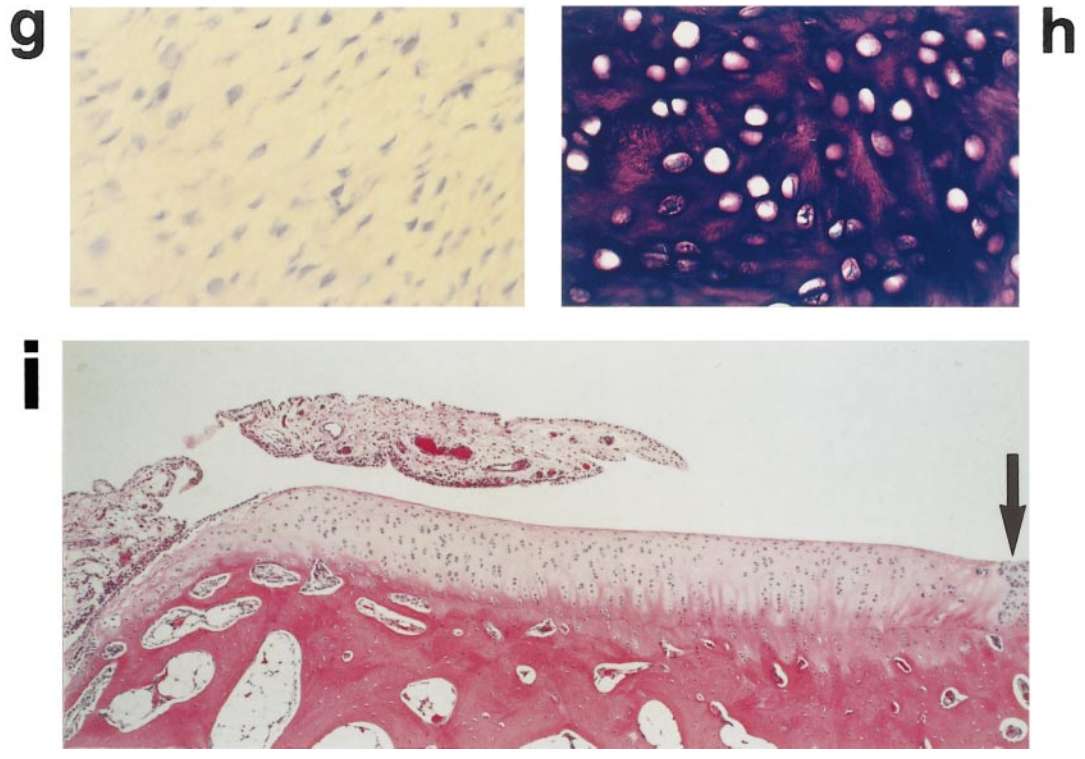

FIG. 3—*Continued*

IGFs, TGF-β_2 and BMP-4 signals (Akiyama, H., submitted). In this study, we demonstrated that TAK-778 markedly stimulates chondrogenesis in chondroprogenitor-like ATDC5 cells *in vitro*. These observations raise the possibility that TAK-778 may promote chondrogenesis of not only chondroprogenitor-like ATDC5 cells *in vitro* but bone marrow-derived mesenchymal cells *in vivo*.

The *in vivo* effect of TAK-778 was tested using a rabbit model of full thickness osteochondral defects in knee joints. TAK-778 possesses the unique activity *in vivo*. Histological studies showed that TAK-778 induced chondrogenesis of mesenchymal cells in osteochondral defects of articular cartilage and dramatically accelerated the articular cartilage repair, while this agent had no adverse effects on the surrounding normal cartilage. Although it remains to be elucidated whether this reparative tissues have cellular characteristics as well as mechanical properties of normal hyaline cartilage, these observations favor the notion that TAK-778 stimulates chondrogenesis via selectively acting on chondroprogenitors in the cartilage lesions.

TGF-β is a potent stimulator of chondrogenesis *in vivo*. Additionally, we recently indicated that TGF-β_2 is required for the onset of chondrogenesis in ATDC5 cells (Akiyama *et al.*, submitted). As shown in Fig. 2, TAK-778 stimulated the expression of TGF-β_2 mRNA in undifferentiated ATDC5 cells, suggesting that stimulatory effect of TAK-778 on chondrogenesis may be mediated by the expression of TGF-β_2 in both ATDC5 cells *in vitro* and chondroprogenitors *in vivo*. Verification of the possibility needs further studies.

The apparent specificity to the repair process of osteochondral defect gives TAK-778 the clinical advantage as a local therapeutic agent over the growth/differentiation factors, such as TGF-βs and BMPs, which also possess chondrogenesis promoting activity. Additionally, the non-invasiveness in administering TAK-778 contrasts sharply to the surgical intervention mandatory to autologous chondrocyte transplantation technique. Thus, successful cartilage repair by topical administration of a synthetic low-molecular agent, TAK-778, selectively acting on chondroprogenitors in cartilage lesions, may open a whole new approach in joint resurfacing.

ACKNOWLEDGMENT

We thank Dr. Y. Hiraki (Kyoto University) for valuable help.

REFERENCES

1. Cuevas, P., Borgos, J., and Baird, A. (1988) *Biochem. Biophys. Res. Commun.* **156,** 611–618.
2. Sellers, R. S., Pelusi, D., and Morris, E. A. (1997) *J. Bone Joint Surg. Am.* **79-A,** 1452–1463.
3. Brittberg, M., Lindahl, A., Nilsson, A., Ohlsson, C., Isaksson, O., and Peterson, L. (1994) *N. Engl. J. Med.* **31,** 889–895.

4. Notoya, K., Yoshida, K., Tsukuda, R., and Taketomi, S. (1994) *J. Bone Miner. Res.* **9,** 395–400.
5. Oda, T., Notoya, K., Gotoh, M., Taketomi, S., Fujisawa, Y., Makino, H., and Sohda, T. (1999) *J. Med. Chem.*, in press.
6. Akiyama, H., Shigeno, C., Hiraki, Y., Shukunami, C., Kohno, H., Akagi, M., Konishi, J., and Nakamura, T. (1997) *Biochem. Biophys. Res. Commun.* **235,** 142–147.
7. Akiyama, H., Hiraki, Y., Shigeno, C., Kohno, H., Shukunami, C., Tsuboyama, T., Kasai, R., Suzuki, F., Konishi, J., and Nakamura, T. (1996) *J. Bone Miner. Res.* **11,** 22–28.
8. Wakitani, S., Imoto, K., Kimura, T., Ochi, T., Matsumoto, K., and Nakamura, T. (1997) *Acta Orthop. Scand.* **68,** 474–480.
9. Shukunami, C., Shigeno, C., Atsumi, T., Ishizeki, K., Suzuki, F., and Hiraki, Y. (1996) *J. Cell Biol.* **133,** 457–468.
10. Shukunami, C., Ishizeki, K., Atsumi, T., Ohta, Y., Suzuki, F., and Hiraki, Y. (1997) *J. Bone Miner. Res.* **12,** 1174–1188.
11. Kulyk, W. M., Rodgers, B. J., Greer, K., and Kosher, R. A. (1989) *Dev. Biol.* **135,** 424–430.
12. Joyce, M. E., Roberts, A. B., Sporn, M. B., and Bolander, M. E. (1990) *J. Cell Biol.* **110,** 2195–2207.