scribed the anteroinferior fasciculi as more robust and shorter than the posterosuperior fasciculi because the period of elongation during development was longer in the posterosuperior fasciculi. This supports our finding that the percentage of type B cases was higher when examining the posterosuperior fasciculi because type B has less collagenous tissue.

A posterolateral meniscal lesion always impedes accurate diagnosis on MRI [18, 19]. One cause of this is the existence of PMF. Although the number of healthy volunteers might be too small to generalize to the general population, our findings will contribute to understanding of the normal appearance of the PMF and will help to determine the proportion of variation in this structure on MRI.

In conclusion, the optimal method for depicting PMF on MRI is the use of proton density–weighted images of 3-mm slice thickness, 256 × 512-matrices, and 45° oblique coronal views. Preferably, T2-weighted, 45° images should be obtained as well. We also inferred from this study that about 30% of healthy knees lack the anteroinferior fasciculi of the PMF and about 50% lack the posterosuperior fasciculi, if we consider PMF to be present only when depicted as a tense low-intensity band on MRI. Even when we defined the existence of PMF by the presence of any structure on MRI, 5–12% of healthy knees may still lack the PMF. Finally, our findings, as presented in this article, will contribute to understanding of the normal appearance of the PMF on MRI, and of the proportion of variation of this structure among the population.

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Mechanism of osteogenic induction by FK506 via BMP/Smad pathways

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Abstract

FK506 is an immunosuppressant that exerts effects by binding to FK506-binding protein 12 (FKBP12). Recently, FK506 has also been reported to promote osteogenic differentiation when administered locally or in vitro in combination with bone morphogenetic proteins (BMPs), although the underlying mechanism remains unclarified. The present study initially showed that FK506 alone at a higher concentration (1 μM) induced osteogenic differentiation of mesenchymal cell lines, which was suppressed by adenoviral introduction of Smad6. FK506 rapidly activates the BMP-dependent Smads in the absence of BMPs, and the activation was blocked by Smad6. Overexpression of FKBP12, which was reported to block the ligand-independent activation of BMP type I receptor A (BMPRIA), suppressed Smad signaling induced by FK506, but not that induced by BMP2. BMPRIA and FKBP12 bound to each other, and this binding was suppressed by FK506. These data suggest that FK506 promotes osteogenic differentiation by activating BMP receptors through interacting with FKBP12.

Keywords: FK506; BMP; Smad; FKBP12; Osteogenic differentiation; Osteoblast

FK506 is an immunosuppressive agent with an increasing number of clinical applications [1–4]. FK506 exerts its immunosuppressive effects by binding to the FK506-binding protein 12 (FKBP12) [5]. The complex of FK506 and FKBP12 inactivates calcineurin, resulting in the inhibition of the cytokine expression including interleukin-2, interleukin-3, and γ-interferon in T cells and the consequent immunosuppression [6]. In addition to its immunosuppressive activity, FK506 has been shown to exert a variety of actions on bone metabolism. When administered systemically, FK506 causes osteopenia in mice, rats, and humans [7–11]. When administered locally or in vitro in combination with bone morphogenetic proteins (BMPs), FK506 promotes osteogenic differentiation [12–14].

BMPs are members of secreted signaling proteins that belong to the transforming growth factor-β (TGF-β) superfamily. BMPs were originally identified as molecules that induced ectopic bone formation when implanted into the rodent muscle [15,16]. In accordance with such in vivo effects, the BMPs have been shown to regulate osteogenic differentiation in vitro [17]. They bind to a characteristic pair of transmembrane serine/threonine kinase receptors, BMP type I and type II receptors (BMPRI and BMPRII). They first bind to the BMPRII, which phosphorylates the GS region of the BMPRI [18]. The activated BMPRI subsequently recruits and phosphorylates Smad1, Smad5, and Smad8 (the BMP-dependent Smads) through the GS region. The BMP-dependent Smads then physically associate with Smad4, translocate into the nucleus, and activate the target genes. Smad6 blocks BMP signaling by inhibiting the phosphorylation of the BMP-dependent Smads by the BMPRI. FKBP12 has been reported to block the ligand-independent activation of the BMPRI [19], but whether FKBP12 mediates the interactions of BMP signaling and FK506 remains unknown. The current study investigated

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the regulation and the mechanism of action of FK506 on osteogenic differentiation of mesenchymal cell lines using the in vitro culture systems.

Materials and methods

In vitro osteogenic differentiation assay. MLB13MYC clone 17 (C17), a limb bud-derived cell line, which differentiates into osteoblasts upon treatment with BMP signaling [20], was a generous gift from V. Rosen (Harvard University, MA). C2C12, a mouse myoblastic cell line, which differentiates into osteoblasts upon treatment with BMP signaling [21], was obtained from the Riken Cell Bank (Tsukuba, Japan). These cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS) (Sigma–Aldrich) and 1% penicillin/streptomycin (Sigma–Aldrich). For the alkaline phosphatase (ALP) staining, the cells were fixed in 70% ETOH and stained for 10 min with a solution containing 0.01% naphthol AS-MX phosphate disodium salt (Sigma–Aldrich), 1% *N,N*-dimethylformamide (Wako Pure Chemicals Industry Tokyo), and 0.06% fast blue BB (Sigma–Aldrich). FK506, cyclosporin A (CsA), recombinant human BMP2 (rhBMP2), and Noggin were purchased from Sigma–Aldrich.

Real-time RT-PCR. The total RNA was extracted using an ISOGEN Kit (Wako) and an RNeasy Mini Kit (QIAGEN, Hilden, Germany), and treated with DNaseI (QIAGEN), according to the manufacturer's instructions. One microgram of RNA was reverse-transcribed using a Takara RNA PCR Kit (AMV) ver.2.1 (Takara, Shiga, Japan) to generate the single-stranded cDNA. PCR was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster-city, CA). Each PCR consisted of 1XQuantiTect SYBR Green PCR Master Mix (QIAGEN), 0.3 μM specific primers, and 500 ng DNA. The mRNA copy number of a specific gene in the total RNA was calculated using a standard curve generated with serially diluted plasmids containing PCR amplicon sequences and normalized to the human or rodent total RNA (Applied Biosystems) with mouse actin as the internal control. The standard plasmids were synthesized using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instruction. All reactions were run in triplicate. The primer sequences are available upon request.

Preparation of adenoviruses and plasmids. Adenoviruses expressing LacZ and Smad6 were generous gifts from K. Miyazono (The University of Tokyo, Tokyo, Japan). Adenoviruses were amplified in HEK293 cells and purified using an AdenoX Virus Purification Kit (Clontech, Palo Alto, CA). Viral titers were determined by the end-point dilution assay. Plasmids expressing the HA-tagged BMP type I receptor A (HA-BMPRIA) and Flag-tagged FKBP12 (Flag-FKBP12), and the luciferase reporter construct responding to BMP-dependent Smad signaling (12xGCCG-luc) were generous gifts from K. Miyazono.

Luciferase assay. The human hepatoma cell line HuH-7 was obtained from the Riken Cell Bank. HuH7 cells were plated onto 24-well plates and then transfected with 0.1 μg of the reporter plasmid construct alone or in combination with the plasmid expressing HA-BMPRIA and Flag-FKBP12 for 1 day, then treated with FK506 (0.1 or 1 μM) or rhBMP2 (200 ng/ml) and cultured for 2 days. The luciferase assay was performed 48 h after transfection using a PicaGene Dual SeaPansy Luminescence Kit (Toyo Ink, Tokyo, Japan) and the Lumat LB 9507 (Berthold Technologies GmbH, KG, Wildbad, Germany). The level of luciferase activity was normalized to the level of the *Renilla* luciferase activity. All data were expressed as means ± SE ($n = 6$).

Immunoblot and immunoprecipitation assay. The cells were washed twice with ice-cold PBS, and the proteins were extracted using an M-PER Kit (Pierce Chemical, Rockford, IL) according to the manufacturer's instructions. The protein concentrations of the cell lysates were measured using a Protein Assay Kit II (Bio-Rad, Hercules, CA). For the immunoblot analysis, the lysates were fractionated by SDS–PAGE with 4–20% Tris–glycine gradient gel or 18% Tris–glycine gel (Invitrogen) and transferred onto nitrocellulose membranes (Bio-Rad). After being blocked with 6% milk/TBS-T, the membranes were incubated with the anti-Smad1 mouse monoclonal antibody (1:1000; Cell Signaling Technology, Beverly MA), anti-phospho-Smad 1/5/8 (1:1000; Cell Signaling Technology), anti-HA mouse monoclonal antibody (1:1000; Santa Cruz Biolaboratories, Santa Cruz, CA), or anti-Flag rabbit antibody (1:1000; Sigma–Aldrich). The secondary antibodies, i.e., HRP-conjugated goat anti-mouse IgG (Promega, Madison, WI) and goat anti-rabbit IgG (Promega), were used at dilutions of 1:10,000. The immunoreactive bands were visualized using an ECL Plus Kit (Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

For immunoprecipitation, 293 cell extracts were incubated with 5 μg of anti-HA and anti-Flag antibodies at 4 °C overnight. The immune complexes were recovered with protein G–Sepharose (Sephadex G-50 Fine; Amersham Life Sciences), subjected to SDS–PAGE, and then transferred onto nitrocellulose membranes. Immunoblotting was performed as already described.

Statistical analysis. Means of groups were compared by ANOVA, and the significance of differences was determined by post hoc testing using Bonferroni's method.

Results

Induction of osteogenic differentiation by FK506

To clarify the effect of FK506 on osteogenic differentiation, we treated the limb bud-derived cell line C17 with FK506 at concentrations ranging from 0.01 to 10 μM. This cell line has been shown to rapidly undergo osteogenic differentiation upon treatment with BMP signaling [20]. Consistent with the report by Tang et al. [12], the treatment with FK506 at 0.1 μM for 3 days induced the ALP activity determined by the ALP staining, but not the osteocalcin mRNA expression determined by the real-time RT-PCR analysis (Figs. 1A and B). When we treated the cells with FK506 at 1 μM, however, both the ALP activity and osteocalcin expression were induced. When the cells were treated with FK506 at 10 μM, neither the ALP activity nor osteocalcin expression was induced (data not shown), probably due to the toxic effect of the drug. In contrast, CsA, an immunosuppressive drug, which also inhibits calcineurin, did not increase the ALP activity or osteocalcin expression at the concentrations tested (Figs. 1A and B). The induction of the ALP activity and osteocalcin expression by FK506 (1 μM) was also seen in C2C12 cells (Figs. 1C and D). These data suggest that FK506 alone is able to induce osteogenic differentiation and that the effect of FK506 on osteogenic differentiation is not dependent on calcineurin signaling.

Interactions of FK506 and BMP signaling during osteogenic differentiation

Since FK506 has been reported to enhance BMP signaling [12], we investigated the interactions of FK506 and BMP signaling. We treated C17 cells with rhBMP2 at various concentrations in the presence or absence of FK506 (1 μM). The ALP activity induced by rhBMP2 was dose-dependent and reached the maximal intensity at 500 ng/ml (Fig. 2A). The ALP activity induced by FK506 alone was more intense than that induced by rhBMP2 at

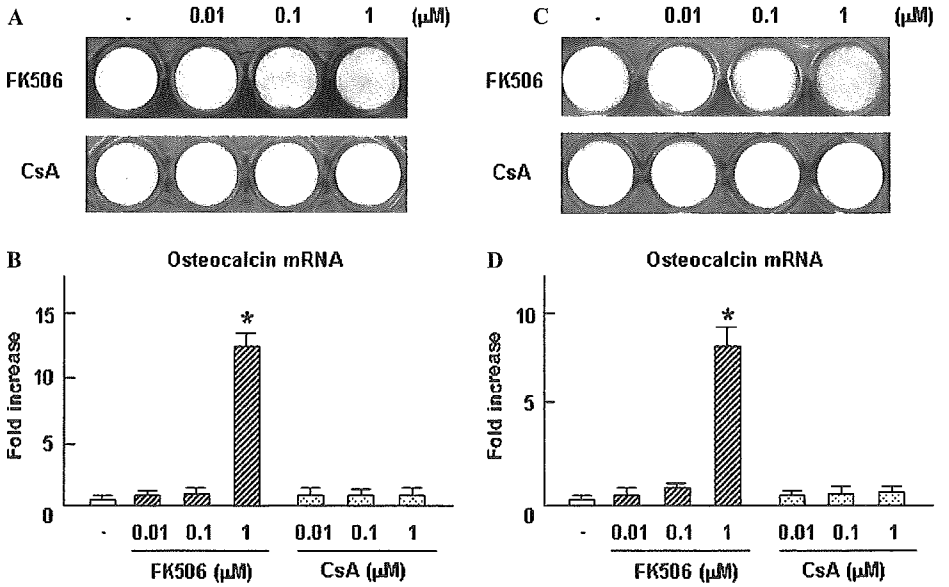

Fig. 1. Induction of osteogenic differentiation by FK506. (A) ALP staining of C17 cells cultured for 3 days in the presence or absence of FK506 or Cyclosporin A (CsA) at various concentrations. (B) Real-time RT-PCR analysis of osteocalcin expression using total RNA isolated from the above-mentioned C17 cells. Data are expressed as means ± SEM of six wells per group. $^*P < 0.01$ vs. no treatment. (C) ALP staining of C2C12 cells cultured for 3 days in the presence or absence of FK506 or CsA at various concentrations. (D) Real-time RT-PCR analysis of osteocalcin expression using total RNA isolated from the above-mentioned C2C12 cells. Data are expressed as means ± SEM of six wells per group. $^*P < 0.01$ vs. no treatment.

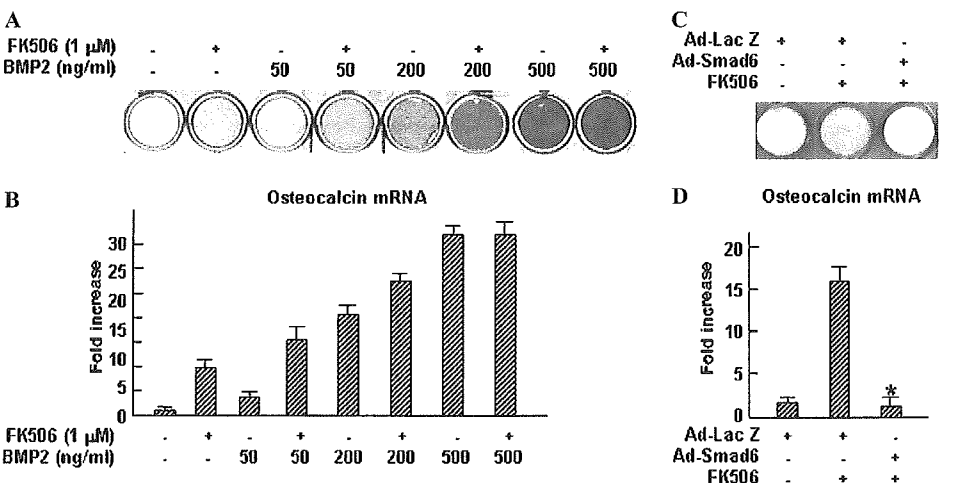

Fig. 2. Interactions of FK506 and BMP signaling in osteogenic differentiation. (A) ALP staining of C17 cells cultured for 3 days in the presence of BMP2 at various concentrations with or without FK506 (1 μM). (B) Real-time RT-PCR analysis of osteocalcin expression using total RNA isolated from the above-mentioned C17 cells. Data are expressed as means ± SEM of six wells per group. $^*P < 0.01$ vs. no treatment. (C) ALP staining of C17 cells infected with an adenovirus expressing LacZ (Ad-LacZ) or an adenovirus expressing Smad6 (Ad-Smad6) for 2 days, and then cultured for 3 days in the presence or absence of FK506 (1 μM). (D) Real-time RT-PCR analysis of osteocalcin expression using total RNA isolated from the above-mentioned C17 cells. Data are expressed as means ± SEM of six wells per group. $^*P < 0.01$ vs. Ad-LacZ+FK506.

50 ng/ml, but less intense than that induced by rhBMP2 at 200 ng/ml. The addition of FK506 to rhBMP2 increased the intensity of the staining by approximately the amount induced by FK506 alone. The real-time RT-PCR analysis of the osteocalcin expression concurred with these data (Fig. 2B). These results suggest that the effect of FK506 on BMP signaling is additive rather than synergistic.

To further investigate whether the osteogenic effect of FK506 required BMP signaling, C17 cells were infected with an adenovirus expressing Smad6 (Ad-Smad6), an inhibitor of BMP signaling, for 2 days and cultured for 3 days in the presence of FK506. The ALP staining and real-time PCR analysis of the osteocalcin expression revealed that the treatment with Ad-Smad6 suppressed

osteogenic differentiation induced by FK506 (Figs. 2C and D).

Activation of BMP-dependent Smads by FK506

Next, we investigated whether FK506 exerted its osteogenic effect by activating the BMP-dependent Smads. The 12xGCCG-luc reporter construct contains the DNA binding site of the BMP-dependent Smads and expresses luciferase in response to BMP signaling [22]. When a human hepatoma cell line HuH-7 was transfected with the 12xGCCG-luc reporter construct in combination with FK506 (0.1 or 1 μM) or rhBMP2 (200 ng/ml), FK506 (1 μM) induced the promoter activity, which was about 60% as strong as that induced by rhBMP2 (Fig. 3A). The transcription of the inhibitor of differentiation-1 (ID-1) has been reported to be dependent on BMP signaling, and its promoter contains a functional DNA binding site for BMP-dependent Smads [23]. When C17 cells were treated with FK506 (1 μM) for 30 min to 5 h, the real-time RT-PCR analysis revealed that FK506 started inducing the ID-1 mRNA expression within a period as short as 30 min, with the expression reaching its peak at 3 h (Fig. 3B). The immunoblot analysis showed that the treatment of C17 cells with FK506 (1 μM) induced the phosphorylation of the BMP-dependent Smads (Fig. 3C). The induction started within a period as short as 30 min, reaching its peak at 1 h and was sustained for up to 5 h.

The results so far suggest that FK506 activates the BMP-dependent Smads in a short period, probably without involving the transcription or translation of the new genes. There are two possible molecular mechanisms. First, FK506 may activate the BMP receptor or the BMP-dependent Smads. Second, FK506 may amplify the action of the existing BMPs. These experiments were carried out in the presence of fetal bovine serum that might contain an undetermined amount of BMPs [24], thus making it difficult to distinguish between these two possibilities. To overcome this problem, C17 cells were cultured in serum-free DMEM with FK506 (1 μM) or rhBMP2 (200 ng/ml) in the presence or absence of simultaneous treatment with Noggin (1000 ng/ml), a potent BMP antagonist. The immunoblot analysis revealed that FK506 induced the phosphorylation of the BMP-dependent Smads in serum-free medium, and that Noggin blocked the

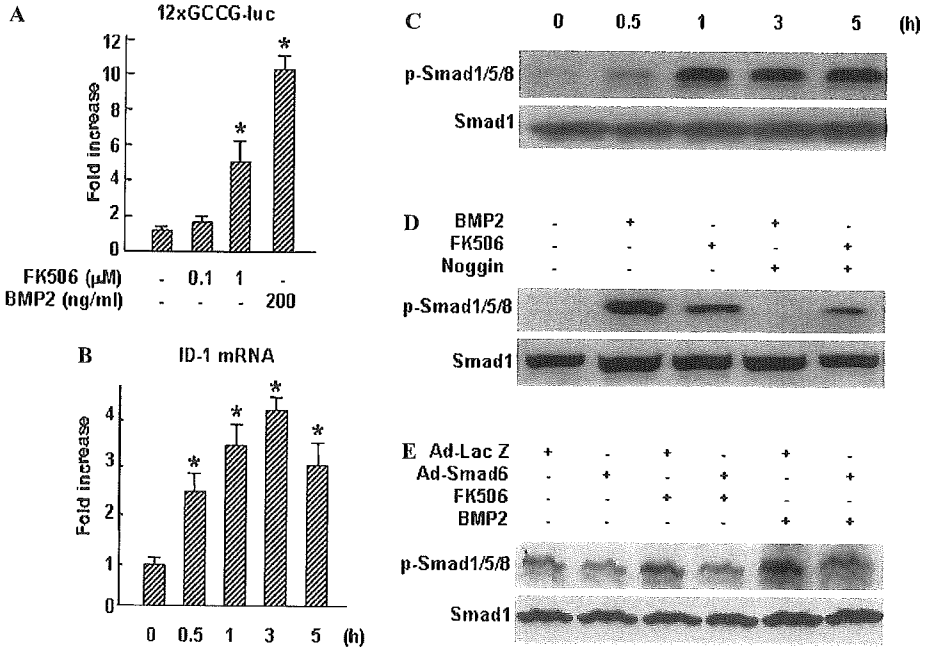

Fig. 3. Activation of the BMP-dependent Smads by FK506. (A) Luciferase reporter analysis of the effect of FK506 on the BMP-dependent promoter activity. Whole cell lysates were collected from HuH-7 cells transfected with the 12xGCCG-luc reporter plasmid construct alone or in combination with FK506 or rhBMP2 and cultured for 2 days. Data are expressed as means ± SEM of six wells per group. $^*P < 0.01$ vs. the reporter plasmid alone. (B) Time course of ID-1 expression determined by real-time RT-PCR using total RNA isolated from C17 cells treated with FK506 (1 μM) for 0.5, 1, 3, and 5 h. Data are expressed as means ± SEM of six wells per group. $^*P < 0.01$ vs. time 0. (C) Time course of induction of Smad1/5/8 phosphorylation by FK506 determined by immunoblot analysis using anti-phospho-Smad1/5/8 antibody (upper panel). Whole cell extracts were collected from C17 cells treated with FK506 (1 μM) for 0, 0.5, 1, 2, 3, 4, and 5 h. The lower panel shows expression of Smad1 to demonstrate normalized loading of proteins. (D) The effect of Noggin on FK506-induced phosphorylation of Smad 1/5/8 determined by immunoblot analysis using an anti-phospho-Smad1/5/8 antibody (upper panels). Whole cell extracts were collected from C17 cells cultured in serum-free DMEM with FK506 (1 μM) or rhBMP2 (200 ng/ml) in the presence or absence of simultaneous treatment with Noggin (1000 ng/ml). The lower panel shows expression of Smad1 to demonstrate normalized loading of proteins. (E) The effect of Smad6 on FK506-induced phosphorylation of Smad 1/5/8 determined by immunoblot analysis using an anti-phospho-Smad1/5/8 antibody (upper panels). Whole cell extracts were collected from C17 cells infected with Ad-Smad6 for 2 days and cultured in the presence of FK506 (1 μM) or rhBMP2 (200 ng/ml) for 1 h. The lower panel shows expression of Smad1 to demonstrate normalized loading of proteins.

phosphorylation of these Smads induced by rhBMP2, but not that induced by FK506. To determine whether FK506 phosphorylates the BMP-dependent Smads through activating the BMP receptors, the effect of Smad6, which binds and inactivates the BMP receptors, on the phosphorylation was investigated. Immunoblot analysis using an anti-phospho Smad1/5/8 antibody revealed that the phosphorylation of Smads by FK506 was suppressed by the adenoviral expression of Smad6 (Fig. 3E). Taken together, these results strongly suggest that FK506 activates the BMP receptors.

Interactions of FK506, the BMP receptors, and FKBP12

In the meantime, FKBP12 has been reported to block the ligand-independent activation of the TGF-β type I receptor, which is homologous to the BMPRI, by binding to the two amino acids of the GS region [25]. FKBP12 has also been reported to block the ligand-independent activation of the BMPRIA [19]. Since the GS region is well conserved between the TGF-β type I receptor and the BMPRI, FK506 may activate the BMPRI by dissociating them from FKBP12. The luciferase reporter analysis revealed that the BMP-dependent promoter activity induced by FK506 was suppressed by the overexpression of FKBP12 (Fig. 4A). The immunoprecipitation and immunoblot analyses revealed that the BMPRIA and FKBP12 bound to each other when they were overexpressed in 293 cells, and this binding was attenuated by treatment with FK506 (Fig. 4B).

To clarify the role of FKBP12 in BMP-induced Smad signaling, the effect of FKBP12 on the BMP-induced Smad phosphorylation was investigated. Immunoblot analysis revealed that overexpression of FKBP12 at various concentrations did not affect the BMP-induced phosphorylation of Smad1/5/8 (Fig. 4C). These data suggest that FKBP12 does not inhibit the BMP-activated BMPRI. Next, we examined the effect of FK506 on the BMP-induced Smad phosphorylation, because FK506 and BMP additively promoted osteogenic differentiation. The immunoblot analysis revealed that BMP-induced phosphorylation of Smad1/5/8 was increased by addition of FK506 in a dose-dependent manner (Fig. 4D).

Discussion

In the current paper, we demonstrate that FK506 alone at a higher concentration induces the osteocalcin expression in the mesenchymal cell lines, while CsA does not;

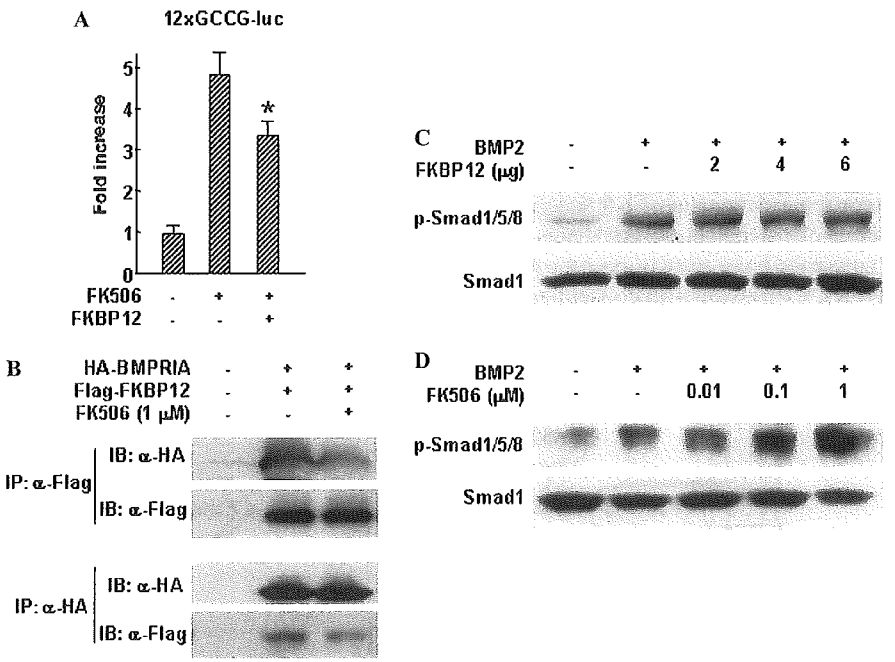

Fig. 4. Interactions of FK506, BMPRI, and FKBP12. (A) Luciferase reporter analysis of the effect of FKBP12 on the FK506-activated BMP-dependent promoter activity. Whole cell lysates were collected from HuH-7 cells transfected with the 12xGCCG-luc reporter plasmid construct alone or in combination with the plasmid expressing HA-BMPRIA and Flag-FKBP12 for 1 day, and then treated with FK506 (1 μM) and cultured for 2 days. Data are expressed as means ± SEM of six wells per group. $^*P < 0.01$ vs. FK506 alone. (B) Physical association of BMPRIA and FKBP12 determined by the immunoprecipitation (IP) and immunoblot (IB) analysis using anti-HA and anti-Flag antibodies. Cell extracts were prepared from 293 cells transfected with or without plasmids expressing HA-BMPRIA and Flag-FKBP12 in the absence or presence of FK506 (1 μM). (C) The effect of FKBP12 on BMP-induced phosphorylation of Smad 1/5/8 determined by immunoblot analysis using an anti-phospho-Smad1/5/8 antibody (upper panel). Whole cell extracts were collected from C17 cells transfected in 6-cm dishes with the plasmid expressing FKBP12 (2, 4 or 6 μg) with rhBMP2 (100 ng/ml). The lower panel shows expression of Smad1 to demonstrate normalized loading of proteins. (D) The effect of FK506 on BMP-induced phosphorylation of Smad 1/5/8 determined by immunoblot analysis using an anti-phospho-Smad1/5/8 antibody (upper panel). Whole cell extracts were collected from C17 cells treated with rhBMP2 (100 ng/ml) in the presence or absence of FK506 at concentrations ranging from 0.01 to 1 μM. The lower panel shows expression of Smad1 to demonstrate normalized loading of proteins.

the induction of the osteocalcin expression by FK506 is blocked by Smad6; the effect of FK506 on BMP signaling is additive rather than synergistic; FK506 rapidly induces the phosphorylation of the BMP-dependent Smads independently of the BMPs, and the induction is blocked by Smad6; and the activation of the Smads by FK506, but not that by BMP2, is attenuated by FKBP12, and the binding of FKBP12 to the BMP receptors is suppressed by FK506.

Although FK506 was reported to induce the ALP activity in some cultured cells and to enhance the osteocalcin mRNA expression induced by the BMPs [12], it was never demonstrated that FK506 alone was able to induce the osteocalcin expression. While the osteocalcin expression is highly specific to osteoblasts, the ALP activity is not. For example, the prehypertrophic and hypertrophic chondrocytes of the growth plate express a considerable amount of ALP [26], but not osteocalcin. Therefore, the effect of FK506 alone on the indisputable osteogenic differentiation remained unclarified. We demonstrated that FK506 alone at a higher concentration (1 μM) was able to induce the osteocalcin mRNA expression.

The high-turnover osteopenia was reported to be caused in mice, rats, and humans by the systemic administration of FK506 at approximately 0.01 μM and that of CsA at approximately 0.1 μM, which are clinical dosages used for the induction of immunosuppression [9,27,28]. At these concentrations, both FK506 and CsA inhibit calcineurin, similarly leading to a reduced function of the T cells [6]. Since FK506 inhibits nuclear factor of activated T cells (NFAT), an essential molecule for osteoclast differentiation, through inactivating calcineurin, FK506 is expected to decrease the number of osteoclasts. Both drugs, however, were reported to increase bone resorption by promoting the stromal cell RANKL expression and consequent osteoclast differentiation [29–31]. Further study is required to clarify this inconsistency. Our results showed that osteogenic differentiation as indicated by the osteocalcin mRNA expression was induced by FK506 at 1 μM, but not by CsA at the concentrations tested, strongly suggesting that this induction is independent of the inhibition of calcineurin and that FK506 at higher concentrations may have an additional downstream signaling pathway for osteogenic differentiation. We demonstrate that BMP signaling is such a pathway.

FK506 exerts its immunosuppressive effects via binding to FKBP12 [5]. We hypothesized that the osteogenic signal induced by FK506 might also involve FKBP12, which was reported to inhibit the ligand-independent signal from the BMPRIA. Our results show that BMPRIA and FKBP12 bound to each other, and that the overexpression of FKBP12 suppressed the activation of Smad signaling by FK506. These data suggest that FK506 may activate BMP signaling by competing for the binding site of FKBP12 with the BMP receptors. Alternatively, FK506 may be able to directly activate the BMP receptors, and FKBP12 may inhibit Smad signaling by sequestering FK506. Since FK506 dissociated FKBP12 from the BMPRIA, we find the first possibility more likely. This competitive interrelationship of FK506 and FKBP12 during osteogenic differentiation contrasts with the cooperative one during immunosuppression, in which FK506 and FKBP12 form a complex and exert the effect. As for the interaction between FK506 and BMP, the BMP2-induced phosphorylation of Smad1/5/8 was increased by addition of FK506. However, overexpression of FKBP12 did not affect the BMP2-induced phosphorylation of Smad1/5/8. We speculate that although FKBP12 blocks the ligand-independent activation of the BMPRI, it may not be able to bind and suppress the BMP-activated BMPRI (see Fig. 5).

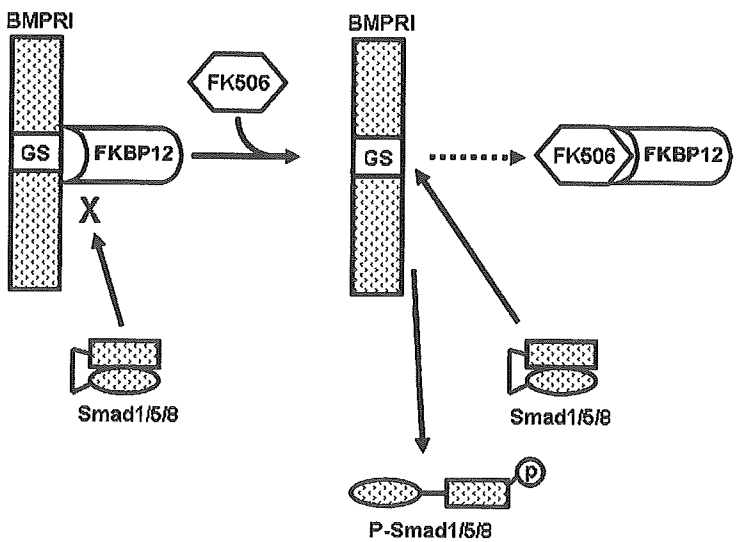

Fig. 5. Schematic representation of BMP signal activation by FK506. Based on the above-mentioned results, we propose the following model. FKBP12 binds to the BMPRI, stabilizes the inactive conformation, and blocks the ligand-independent activation of the receptors (left). FK506 competes for the GS region of the BMPRI with FKBP12 and allows the ligand-independent phosphorylation of the BMP-dependent Smads (right).

Recently, it has been reported that FK506 inhibits the osteoblast differentiation and function by suppressing expression of Runx2 and osterix mRNAs in ROS 17/2.8 cells [32] and by attenuating osterix function through NFAT signaling without changing expression of Runx2 mRNA [9]. These complex and confusing effects on the osteogenic differentiation by FK506 may be derived from the compound effects of FK506 on NFAT and BMP signalings. Although FK506 at the concentrations for immunosuppression mainly inhibits ostegenic differentiation by attenuating osterix function through regulating NFAT [9], FK506 at higher concentrations induces osteogenic differentiation by stimulating BMP/Smad signaling through regulating the interactions between BMPRI and FKBP12.

In conclusion, FK506 promotes osteogenic differentiation by activating the BMP receptors, probably through segregating them from FKBP12. In bone regenerative medicine, BMPs have been clinically used for treating intractable fractures and for spinal fusion [33–35]. The treatment, however, requires a large amount of expensive recombinant BMPs, thus hindering this method from becoming widely available. The osteogenic effect of FK506 at 1 μM roughly corresponds to that of BMP at 100 ng/ml. Provided that its systemic adverse effect can be avoided, the local use of FK506 as a surrogate for BMPs may help reduce the cost and may help broaden the application of bone regenerative medicine.

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Synergistic Effects of FGF-2 With Insulin or IGF-I on the Proliferation of Human Auricular Chondrocytes

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Chondrocyte preparation with the safety and efficiency is the first step in cartilage regenerative medicine. To prepare a chondrocyte proliferation medium that does not contain fetal bovine serum (FBS) and that provides more than a 1000-fold increase in cell numbers within approximately 1 month, we attempted to use the medium containing 5% human serum (HS), but it exerted no more than twofold increase in 2 weeks. To compensate for the limited proliferation ability in HS, we investigated the combinational effects of 12 factors [i.e., fibroblast growth factor(FGF)-2, insulin-like growth factor(IGF)-I, insulin, bone morphogenetic protein-2, parathyroid hormone, growth hormone, dexamethasone, 1α25-dihydroxy vitamin D_3, L-3,3′,5′-triodothyronine, interleukine-1 receptor antagonist, 17β-estradiol, and testosterone] on the proliferation of human auricular chondrocytes by analysis of variance in fractional factorial design. As a result, FGF-2, dexamethasone, insulin, and IGF-I possessed promotional effects on proliferation, while the combination of FGF-2 with insulin or IGF-I synergistically enhanced the proliferation. Actually, the chondrocytes increased 7.5-fold in number in 2 weeks in a medium containing 5% HS with 10 ng/ml FGF-2 , while the cell number synergistically gained a 10–12-fold increase with 5 μg/ml insulin or 100 ng/ml IGF-I in the same period. The proliferation effects were more enhanced at a concentration of 100 ng/ml for FGF-2, and especially for the combination of 100 ng/ml FGF-2 and 5 μg/ml insulin (approximately 16-fold within 2 weeks). In the long-term culture with repeated passaging, this combination provided more than 10,000-fold within 8 weeks (i.e., passage 4). Thus, we concluded that such a combination of FGF-2 with insulin or IGF-I may be useful for promotion of auricular chondrocyte proliferation in a clinical application for cartilage regeneration.

Key words: Chondrocyte; Proliferation; Regenerative medicine; Medium; Soluble factor; Fractional factorial design

INTRODUCTION

Tissue engineering is a challenging technology in which the tissues or the cells are cultured in the laboratory and are used for replacement or support of the function of defective or injured body parts. This new approach is anticipated to overcome the difficulties or problems in the present clinical treatment. Recently, the studies on tissue engineering have endeavored to grow every type of human tissue: liver, bone, muscle, carti-

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lage, blood vessels, heart muscles, nerves, pancreatic islets, and more. Among them, cartilage regenerative medicine has progressed well. Tissue-engineered cartilage has already been available for clinical use in the treatment of patients with joint defects (5,22,23) or for the correction of vesicoureteral reflux (6).

However, to broaden the indication range of cartilage regenerative medicine (e.g., to microtia, which is a congenital anomaly of the ear, cleft lip and palate, or osteoarthritis), some improvement would be necessary. One of issues to be improved is cell preparation with safety and efficiency. We should obtain a sufficient cell number from a small volume of specimen within a limited period, for clinical use. In a previous report on autologous chondrocyte transplantation for joint defects, a culture medium with 10% autologous human serum provided approximately 5×10^6 cells within 3 weeks, corresponding to 0.3–0.4 ml of regenerative cartilage material (23). However, because the volume of the regenerative cartilage needed in the treatment of microtia or end-stage osteoarthritis of knees may be some tens of milliliters, more than a 100-fold increase in cell numbers would need to be prepared for such cases, compared with that in an autologous transplantation.

To improve the efficiency of cell preparation, many researchers have attempted to use growth factors with fetal bovine serum (FBS). Quatela and coworkers examined the proliferation of human auricular chondrocytes in DMEM containing 5% FBS with 10 ng/ml fibroblast growth factor (FGF)-2 and 3 ng/ml transforming growth factor (TGF)-β, which exerted a maximal synergistic effect on thymidine uptake (25).

Although FBS contains various factors that can increase chondrocyte proliferation (7,10), the use of FBS may be restricted for clinical application because it includes the risk for transmission of viral and other pathogens. In addition, problems of possible immune reaction against bovine protein in the serum should be considered when the regenerated tissues cultured in the FBS-contained medium are transplanted into humans. The previous studies had shown immune response by antibody detection against bovine serum proteins in burn patients receiving keratinocyte grafts cultured from FBS (12,19).

Under clinical conditions, we may use human serum (HS) obtained from the patients by autologous blood transfusion, which would help the proliferation of their own cells. With HS, we statistically examined the additional effectiveness of soluble factors with regard to their synergy in chondrocytes obtained from the patients. For this experiment, we chose 12 kinds of soluble factors, all of which possess some effect on chondrocyte proliferation or differentiation and are clinically available because their safety has already been secured. Using the statistical method that is termed "analysis of variance by fractional factorial design" (8), the effects of the individual factors and the synergy of combinations were evaluated. This statistical method is useful when the number of potential factors is large, because it can minimize the total numbers of runs required. According to this method, we selected highly effective combinations of soluble factors for proliferation of human auricular chondrocytes, which might be immediately applied in the clinical field.

MATERIALS AND METHODS

Growth Factors and Reagents

Dulbecco's modified Eagle's medium (DMEM), DMEM nutrient mixture F-12 HAM (DMEM/F12), penicillin-streptomycin solution, trypsin-EDTA solution, fetal bovine serum (FBS), and human serum (HS, lot# 043K0500, 043K0501, and 043K0502) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dexamethasone (Dex), 1α25-dihydroxy vitamin D_3 (vitD), L-3,3′,5′-triiodothyronine (T3), and 17β-estradiol (E_2) were from EMD Bioscience (San Diego, CA, USA). Collagenase from *Clostridium histolyticum* and ISOGEN were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other reagents were: atelocollagen (Kawaken Fine Chemicals Co., Ltd., Tokyo, Japan), bullet kit chondrocyte growth medium (CGM, Cambrex Bioscience Walkersville, Inc., Walkersville, MD, USA), FGF-2 (kindly provided by Kaken Pharmaceutical Corporation, Ltd., Tokyo, Japan), recombinant human insulin-like growth factor-I (IGF-I, former Genzyme-Techne, Minneapolis, MN), insulin (MP Biomedicals Inc., Irvine, CA, USA), recombinant human bone morphogenetic protein-2 (BMP-2, kindly provided by Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan), human parathyroid hormone [PTH (1–34), Anaspec, Inc., San Jose, CA], human growth hormone (GH, Biogenesis, Ltd., Poole, UK), recombinant human interleukin-1 receptor antagonist (IL-1 RA, Strathmann Biotech GmbH, Hamburg, Germany), and testosterone (Ultrafine Chemicals, Manchester, UK).

Cell Isolation

All procedures of the present experiments were approved by the ethic committee of the University of Tokyo Hospital (ethic permission #622). Remnant auricular cartilage and surgical debris of the costal cartilage were obtained from five children (age range 10–15 years) who underwent microtia surgery at the University of Tokyo Hospital, with informed consent. The perichondrium was separated from the auricular cartilage under sterile conditions, while that of the costal cartilage had been already removed during the operation. The isolated cartilage was minced into 1-mm^3 pieces and digested with 0.15% collagenase in DMEM containing penicillin and streptomycin at 37°C for 24 h. The digested suspen-

sion was filtered using a sterile 100-μm nylon cell strainer (BD Falcon, Bedford, MA, USA) and centrifuged at 430 × *g* for 5 min. The resulting pellet of cells was washed twice with DMEM containing the antibiotics and then resuspended in the medium. The number of cells was calculated using a hemocytometer, and the viability of the cells was determined using trypan blue vital dye.

Chondrocyte Culture and Evaluation of Proliferation

Chondrocytes were suspended in 0.24% atelocollagen solution (pH 7). The mixture was placed in each well of a six-well plate at 2 ml, or a 96-well plate for 0.1 ml, at a density of 2×10^4 cells/ml. The atelocollagen formed a gel in 1-h incubation at 37°C, embedding the cells in a 3D condition. The commercial medium, CGM containing 5% FBS with an undisclosed concentration of FGF-2, IGF-I, and insulin, or the medium containing DMEM/F-12 with or without serum and/or soluble factors was gently poured on the gel at a volume of 2 ml or 0.1 ml, respectively, in a 37°C/5% CO_2 incubator. Throughout the experiment, the medium was changed three times per week. To release the cells, the gel was incubated in 0.3% collagenase at 37°C for 2 h.

To evaluate the cell proliferation by cell count, the auricular chondrocytes (cultured in CGM, passage 4) from the five patients were individually incubated in five wells of a six-well plate for each medium. After a 2-week incubation, the cell numbers were counted by a hematocytometer, while the viability of cells was checked by trypan blue staining. For the long-term culture with repeated passaging of the auricular or costal chondrocytes, we cultured the cells in three wells of six-well plates with the CGM or the DMEM/F-12 with 5% HS and soluble factors. Passage was performed every week or every other week, while the mean cell numbers in the three wells were counted during every passage.

To evaluate the samples on the 96-well plates, we used a colorimetric assay for cell proliferation, Cell Proliferation Kit II (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, after the release of chondrocytes from the gel in each well of the 96-well plates, the cells were centrifuged for 5 min at 430 × *g* and separated from the supernatant, which was then removed. The labeling mixture was then added to the cells, which were incubated for 4 h in a 37°C/5% CO_2 incubator. The spectrophotometric absorbance of the samples using a microtiter plate reader was measured at a wavelength of 450 nm. The reference wavelength was 650 nm.

Fractional Factorial Design

Twelve factors (i.e., FGF-2, IGF-I, insulin, BMP-2, PTH, GH, Dex, vitD, T3, IL-1RA, E_2, and testosterone) were examined for their combinational effects on proliferation. The doses of each factor were used from previous papers or were determined as their modifications due to our own preliminary data and/or economical reasons (Table 1). With the usual factorial design, $2^{12} = 4096$ treatment combinations would be needed, but this is practically impossible. We adopted fractional factorial design (8) to reduce the experimental units to a practical number of 256 combinations. This design retains good statistical properties and has found its greatest use in industrial research. We used the software JMP-5.1.1J (SAS Institute, Inc., Cary, NC, USA) to generate the 256 kinds of randomized combinations in which those 12 factors independently appeared on the incidence of 50% (Table 2). We then made the same number of media containing DMEM/F-12 with those combinations of factors and 5% HS. Three kinds of auricular chondrocytes obtained from different patients were incubated in each well of 96-well plates with those media for 2 weeks. The effects of each medium on proliferation were examined in the colorimetric assay for cell proliferation. Values for three kinds of auricular chondrocytes in each media were processed by analysis of variance using the software JMP, providing an *F*-value, meaning the effects of the individual factors or the interaction terms of two factors, as well as a parameter estimate indicating expected changes produced by one factor or two. Total effect of two factors on proliferation was compared with each other, as the sum of parameter estimates of two main effects and interaction.

Total RNA Extraction and Real-Time PCR

The chondrocytes were released from the atelocollagen gel by a 30-min incubation in 0.3% collagenase. The total RNA was isolated from the chondrocytes with ISOGEN following the supplier's protocol. Complementary DNA (cDNA) was synthesized from 1 μg of total

Table 1. Twelve Soluble Factors Affecting Chondrocyte Proliferation and Differentiation

Soluble Factors	Doses	References
FGF-2	10 ng/ml	13
IGF-I	100 ng/ml	30
Insulin	5 μg/ml	14
BMP-2	200 ng/ml	27
PTH	5×10^{-8} M	32
GH	100 ng/ml	20
Dex	10^{-7} M	21
VitD	10^{-7} M	11
T3	10^{-7} M	17
IL-1 RA	20 ng/ml	31
E_2	10^{-7} M	26
Testosterone	10^{-6} M	18

Table 2. Combinations of Soluble Factors Determined by Fractional Factorial Design F, FGF-2; I, IGF-I; In, insulin; B, BMP-2; P, PTH; G, GH; D, Dex; V, vitD; T, T3; IL, IL-1 RA; E, E_2; Te, testosterone.

	F	I	in	B	P	G	D	V	T	IL	E	Te
1	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	−	−	−	−	−
3	+	+	+	+	+	+	−	+	−	+	+	−
4	+	+	+	+	+	+	−	+	−	+	−	+
5	+	+	+	+	+	+	−	−	−	−	+	+
6	+	+	+	+	+	−	+	+	−	−	−	+
7	+	+	+	+	+	−	+	−	+	−	+	−
8	+	+	+	+	+	−	−	−	+	+	−	+
9	+	+	+	+	−	+	+	+	+	−	−	−
10	+	+	+	+	−	+	+	+	−	+	+	+
11	+	+	+	+	−	+	−	+	+	+	+	−
12	+	+	+	+	−	+	−	−	+	+	−	−
13	+	+	+	+	−	+	−	−	−	−	+	−
14	+	+	+	+	−	−	+	+	+	+	−	−
15	+	+	+	+	−	−	+	+	+	−	+	+
16	+	+	+	+	−	−	+	−	−	+	+	+
17	+	+	+	+	−	−	+	−	−	−	−	+
18	+	+	+	−	+	+	+	+	+	+	+	−
19	+	+	+	−	+	+	+	+	+	−	−	−
20	+	+	+	−	+	+	−	+	−	−	+	+
21	+	+	+	−	+	+	−	−	+	+	+	+
22	+	+	+	−	+	−	+	+	−	+	+	+
23	+	+	+	−	+	−	−	+	+	−	+	−
24	+	+	+	−	+	−	−	+	−	−	−	−
25	+	+	+	−	+	−	−	−	+	−	+	−
26	+	+	+	−	+	−	−	−	−	+	−	+
27	+	+	+	−	−	+	+	−	+	+	+	−
28	+	+	+	−	−	+	+	−	+	−	−	+
29	+	+	+	−	−	+	+	−	−	+	−	+
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32	+	+	+	−	−	−	+	−	−	+	−	−
33	+	+	+	−	−	−	−	+	+	−	−	+
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35	+	+	−	+	+	+	+	+	+	+	−	−
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38	+	+	−	+	+	+	−	+	+	−	−	−
39	+	+	−	+	+	+	−	−	+	−	−	−
40	+	+	−	+	+	−	+	+	−	+	−	−
41	+	+	−	+	+	−	−	−	+	−	+	+
42	+	+	−	+	+	−	−	−	−	+	+	−
43	+	+	−	+	−	+	+	−	+	−	+	−
44	+	+	−	+	−	+	+	−	−	+	−	+
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255	−	−	−	−	−	−	−	−	+	−	−	+
256	−	−	−	−	−	−	−	−	−	+	+	−

RNA with the Superscript II reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA). For the real-time PCR, the ABI Prism Sequence Detection System 7000 was used. Primers were designed based on the sequences obtained from the GenBank, and amplicons of 50–250 base pairs with a melting temperature of between 55°C and 60°C were selected. Aliquots of the first-strand cDNA (1 μg) were amplified with the QuantiTect SYBER Green PCR Kit (Qiagen, Osaka, Japan) under the following conditions: initial denaturation for 10 min at 94°C followed by 40 cycles consisting of 15 s at 94°C and 1 min at 60°C. The data analysis consisted of a fold induction, and the expression ratio was calculated from the differences in the threshold cycles at which an increase in the reporter fluorescence above a baseline signal could first be detected among the three samples and was averaged for duplicate experiments. The sequence of primers we utilized in the real-time PCR to detect human Col2A1 and human GAPDH were: hCol2A1 forward 5′-GAGT CAAGGGTGATCGTGGT-3′, reverse 5′-CACCTTGG TCT CCAGAAGGA-3′; hGAPDH forward 5′-GAAGG TGAAGGTCGGAGTCA-3′, reverse 5′-GAAGATGGT GATGGGATTTC-3′. hGAPDH was used as a housekeeping gene.

RESULTS

Proliferation of Auricular or Costal Chondrocytes With or Without FBS

In a microtia operation, both remnant auricular cartilage and surgical debris of the costal cartilage, neither of which is used for reconstructive surgery, were obtained from individual patients. The mean weight was 1.18 g (1.00–1.42, $n = 5$) or 3.60 g (2.2–5.0, $n = 5$), respectively, while the mean cell number of the isolated chondrocytes was 3.8×10^6 (1.5×10^6–7.0×10^6) or 2.5×10^6 (1.0×10^6–4.0×10^6). We first examined the proliferation of the auricular and costal chondrocytes taken from these tissues. When the commercial medium, CGM containing 5% FBS with an undisclosed concentration of FGF-2, IGF-I, and insulin, was used for chondrocyte proliferation and passage was performed every week, both kinds of chondrocytes showed exponential growth after some lag phase in all five patients. The growth rate was higher in the auricular chondrocytes than in costal ones. Figure 1 shows the typical growth curve of two patients. The auricular chondrocytes increased their number by 1000-fold within passages 3–4 (3–4 weeks), while it took more than five passages (5 weeks) for the costal ones (Fig. 1).

Next, we attempted to substitute FBS for HS and/or some other factors. When we examined the proliferation of the auricular chondrocytes in the medium without any serum or soluble factors, they did not undergo proliferation at all (Fig. 2, open bar). When we added FGF-2 and IGF-I, whose combination had been known to increase the proliferation in a previous paper (42), little proliferation was noted (Fig. 2, open bar). In contrast, HS may have some effects on the proliferation of auricular chondrocytes, but it provided no more than twofold in 2 weeks (Fig. 2, filled bar). FBS showed better proliferation of approximately threefold within 2 weeks (Fig. 2, filled bar). When FGF-2 and IGF-I were added to the medium containing HS or FBS, the proliferation was increased to approximately seven- to ninefold in both (Fig 2, slanted striped bars), suggesting the possibility that HS may promote proliferation to the same extent as FBS, with some soluble factors. However, none of them were over that of CGM (Fig. 2, horizontal striped bar).

Statistical Analysis for Combinational Effects of Soluble Factors

We selected 12 kinds of soluble factors to be examined (Table 1). We used a fractional factorial design to determine an optimal combination with a minimal number of experiments. Using 256 kinds of media determined by the software JMP-5.1.1.J (Table 2), we examined the proliferation effects in a colorimetric assay. The results obtained for the 256 kinds of media were also analyzed by the software JMP-5.1.1.J.

In this analysis, FGF-2, Dex, insulin, and IGF-I showed parameter estimates of 0.192 ($F = 676$), 0.0943 ($F = 166$), 0.0626 ($F = 75.1$), and 0.0524 ($F = 52.6$), respectively, and they showed significant effects on proliferation alone ($p < 0.001$). Eight other factors did not prominently promote proliferation of the auricular chondrocytes in the presence of 5% HS, and especially BMP-2 worked rather negatively in the chondrocyte proliferation (parameter estimate −0.194, $F = 720$). Regarding synergy, the interaction term of FGF-2 and insulin and that of FGF-2 and IGF-I exhibited statistically positive effects (parameter estimates 0.0582 and 0.0565, $F = 64.3$ and 60.7, $p < 0.001$), although IGF-I and insulin decreased the effects of synergy (parameter estimate −0.0601, $F = 68.7$, $p < 0.001$). Therefore, we chose the combinations of FGF-2 with insulin or IGF-I as ideal ones that showed high F-values in both individual factors and interaction terms of two factors. Actually, the combinations of FGF-2 and insulin or FGF-2 and IGF-I showed high values of parameter estimates, suggesting strong effects on proliferation, when the chondrocytes were cultured together with 5% HS (Fig. 3).

Effects of Doses of FGF-2, Insulin, and IGF-I on the Synergy

To confirm the effects of FGF-2, insulin, IGF-I, or their combinations on chondrocyte proliferation, the increase in cell numbers was counted, following the culture in the media containing 5% HS with those factors

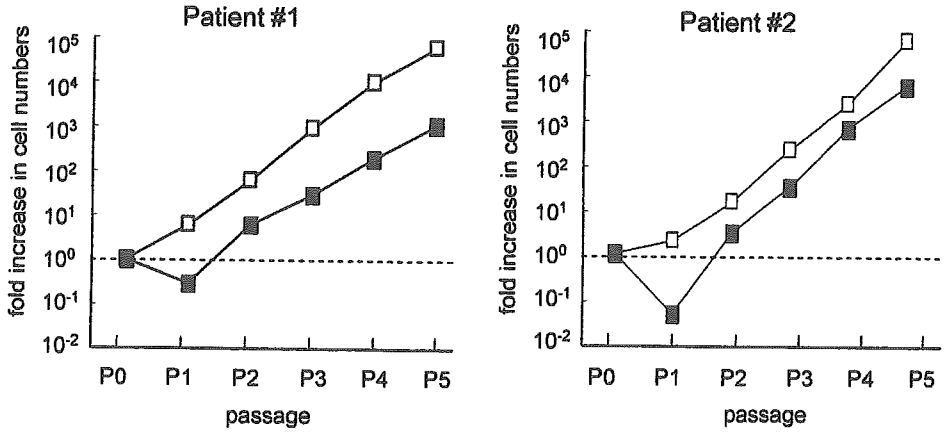

Figure 1. Growth curves of human chondrocytes. Each kind of cell was cultured in a six-well plate with CGM. Every week, the cells were harvested, counted in numbers, and reseeded in a six-well plate until the cell numbers increased more than 1000-fold. The number of auricular chondrocytes (open square) arrived at a 1000-fold increase more quickly than that of the costal cartilage (filled square) in all patients ($n = 5$).

or their combination in various doses. FGF-2 showed a dose dependency for proliferation in the range of 10–500 ng/ml, although more than 1 μg /ml seemed to be the top limit. Insulin and IGF-I did not exhibit increasing effects on proliferation in the range of 5–500 μg/ml or 100 ng–10 μg/ml, respectively (Fig. 4).

The synergistic effects of insulin or IGF-I with FGF-2 seemed to be maintained until the dose of FGF-2 increased to 100 ng/ml (Fig. 5). In the combination of 10 ng/ml FGF-2 with 5 μg/ml insulin or 100 ng/ml IGF-I, the cell number increased to approximately 12- or 10-fold, respectively, within 2 weeks, while the increase was limited to eightfold with FGF-2 alone (Fig. 5, FGF-2 10 ng/ml). The addition of both insulin and IGF-I to FGF-2 showed an 11-fold increase, which was somewhat lower, compared with the combination of FGF-2 with insulin (12-fold) (Fig. 5, FGF-2 10 ng/ml). The same tendency was observed in the combination of 100 ng/ml FGF-2 with insulin, IGF-I, or both. Especially the combination of 100 ng/ml FGF-2 and insulin showed prominent proliferation (16-fold) (Fig. 5, FGF-2 100 ng/ml). When the dose of FGF-2 was 500 ng/ml, the addition of insulin, IGF-I, or both made little difference compared with FGF-2 alone, suggesting that synergistic effects of insulin and IGF-I were not found at this dose of FGF-2 (Fig. 5, FGF-2 500 ng/ml). Although 500 ng/

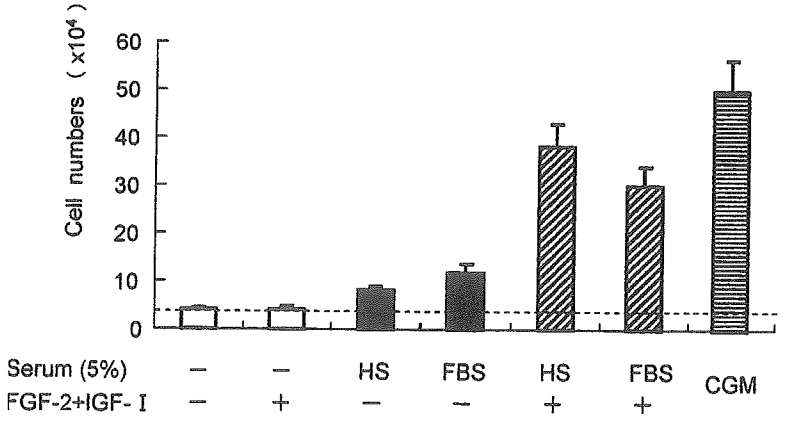

Figure 2. Effects of FBS, HS, or additional soluble factors on the chondrocyte proliferation. Human auricular chondrocytes (2×10^4 cells corresponding to the broken line) obtained from five patients were encapsulated in 2 ml of atelocollagen gel and incubated with each kind of medium in a six-well plate for 2 weeks ($n = 5$ for each medium). HS, human serum; FBS, fetal bovine serum; FGF-2, 10 ng/ml; IGF-I, 100 ng/ml. All values are presented as mean + SD.

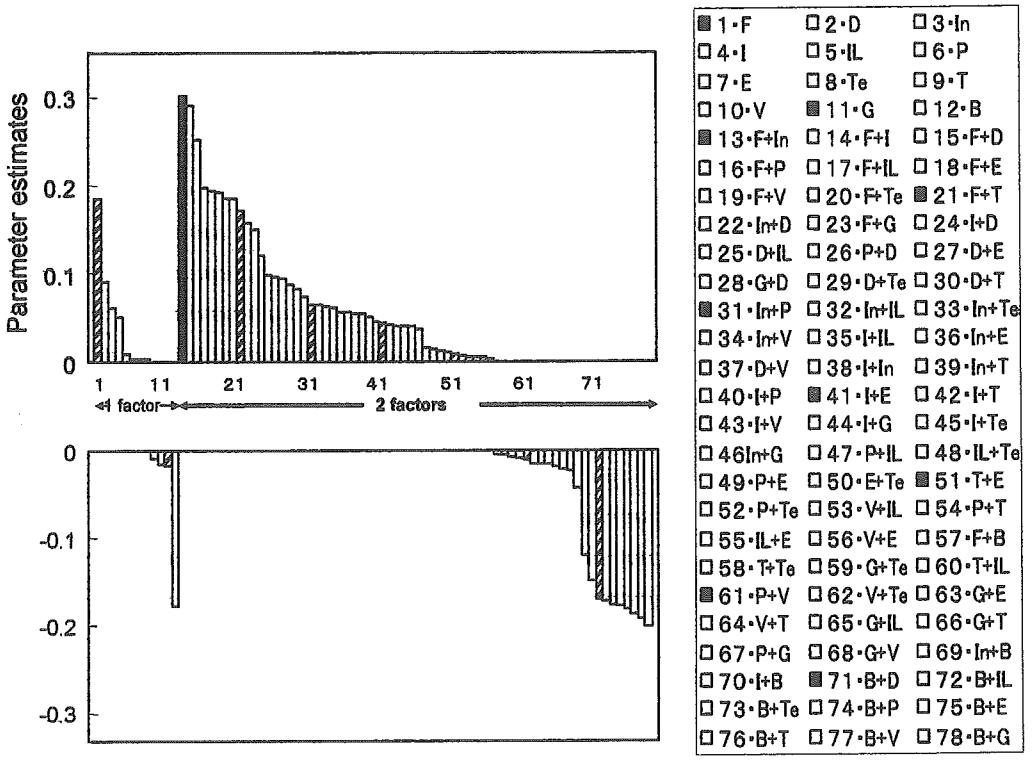

Figure 3. Results of analysis of variance by fractional factorial design. FGF-2 (column #1), Dex (column #2), insulin (column #3), and IGF-I (column #4) showed high values of parameter estimates, suggesting the promotion of proliferation. The combination of FGF-2 and insulin (column #13) and that of FGF-2 and IGF-I (column #14) were strongly effective for proliferation. However, the simultaneous use of insulin and IGF-I (column #38) somewhat decreased the effects of synergy, compared with a single use of insulin (column #3) or IGF-I (column #4). F, FGF-2; D, Dex; In, insulin; I, IGF-I; IL, IL-1RA; P, PTH; E, E_2; Te, testosterone; T, T3; V, vitD; G, GH; B, BMP-2.

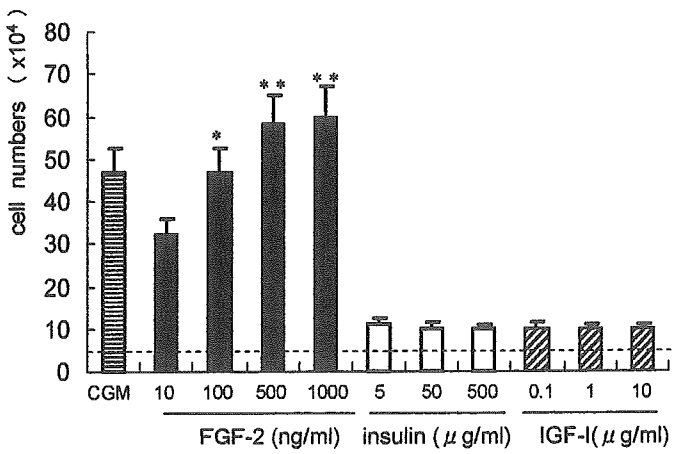

Figure 4. Dose dependency of FGF-2, insulin, and IGF-I in the chondrocyte proliferation. Human auricular chondrocytes were 3D cultured in the media containing DMEM/F-12 with 5% HS and FGF-2, insulin, or IGF-I at various kinds of doses for 2 weeks ($n = 5$ for each medium). All values are presented as mean + SD. Statistics were assessed using the Student's t-test ($^*p < 0.05$ vs. FGF-2 10 ng/ml, $^{**}p < 0.05$ vs. FGF-2 100 ng/ml). The broken line indicates the number of chondrocytes at the starting point of incubation (2×10^4 cells).

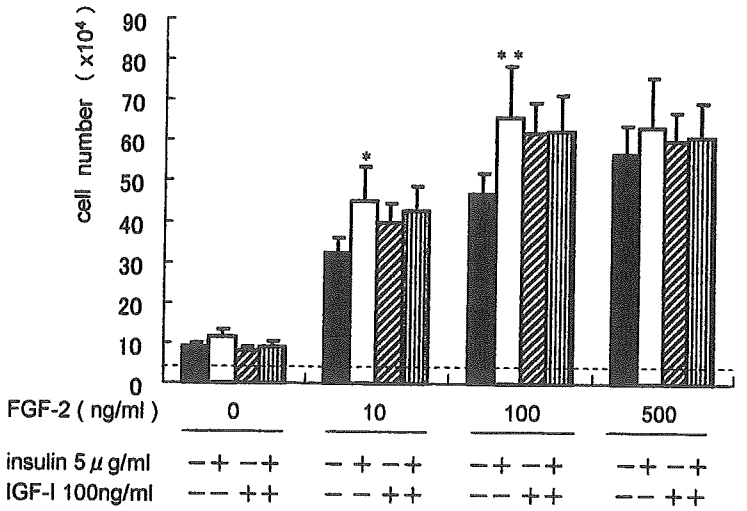

Figure 5. Synergistic effects of FGF-2 with insulin or IGF-I on the chondrocyte proliferation. The cell numbers of human auricular chondrocytes from the different patients were counted after a 2-week incubation ($n = 5$ for each medium). The media contained DMEM/F-12 with 5% HS and the soluble factors. The broken line indicates the initial number of chondrocytes at the starting point (2×10^4 cells). All values are presented as mean + SD. Statistics were assessed using the Student's t-test (*$p < 0.05$ vs. the group using FGF-2 10 ng/ml alone, **$p < 0.05$ vs. the group using FGF-2 100 ng/ml alone).

ml of FGF-2 alone showed higher proliferation effects than did 100 ng/ml, the combinations of 500 ng/ml FGF-2 with insulin, IGF-I, or both showed almost the same proliferation ability as those combinations with 100 ng/ml FGF-2 (Fig. 5). These results were reproducible in every lot of HS (data not shown).

To confirm the actual 1000-fold increase in the cell numbers, we examined a long-term culture with repeated passaging of the auricular chondrocytes in 5% HS-DMEM/F-12 containing FGF-2 (100 ng/ml) with or without insulin (5 μg/ml) or IGF-I (100 ng/ml). A passage was performed every other week, an interval that corresponded to the period used in the above experiments for determining the kinds and doses of the soluble factors. In the growth curves, all kinds of media as well as the CGM provided good proliferation after some lag phase, and realized a 1000-fold increase during passage 4 (Fig. 6). Especially, the combination of FGF-2 and insulin exhibited the highest growth rate, arriving at 10,000-fold within passage 4.

Finally, we checked the gene expression pattern of the chondrocytes proliferated in these four kinds of media. All of them showed an absolutely lower expression in Col2A1, compared with chondrocytes of the primary culture (passage 0). The expression ratio was 7470 ± 2450 in the primary culture, while those of a long-term culture in CGM, the medium containing FGF-2, and that in the combination of FGF-2 with insulin or IGF-I were 2.96 ± 1.82, 7.26 ± 4.59, 11.3 ± 7.11, and 15.5 ± 3.50, respectively.

DISCUSSION

The first issue that we must achieve is to gain sufficient cell numbers of cultured chondrocytes for the cartilage regenerative medicine. A previous report described that fetal elastic chondrocytes proliferated more quickly than fetal hyaline cartilage (9). In the present study, the auricular chondrocytes of school children age patients also had more favorable proliferation, compared with the costal ones. In addition, we could determine an effective proliferation medium containing human serum or several soluble factors, showing high responsiveness to the auricular chondrocytes. Also because the auricular cartilage can be accessed easily in minimally invasive fashion, through a simple auricle biopsy, this kind of chondrocyte would be a good candidate for a cell source for cartilage regenerative medicine.

In the present study, we determined the combinations of two factors, which promote the proliferation of human auricular chondrocyte in the presence of 5% HS, among 12 soluble factors, by analysis of variance in fractional factorial design. For the analysis, we did not confine the factors to be examined to those that had been previously published to be effective for the proliferation of the human auricular chondrocytes, but used many factors that are reported to possess some effects on either

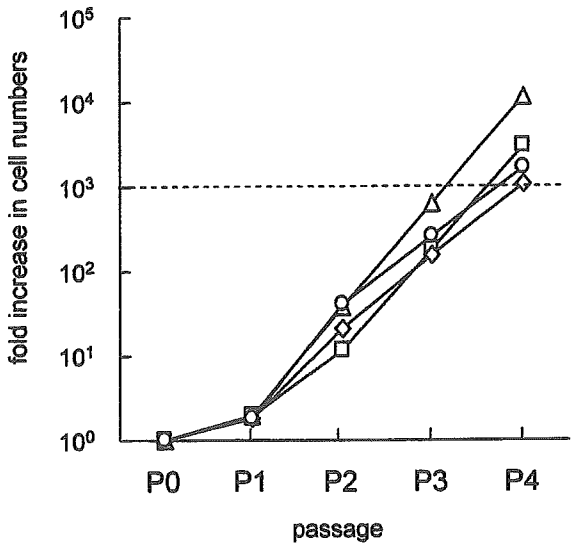

Figure 6. Growth curves of human auricular chondrocytes cultured in various kinds of media. Passage was performed every other week. DMEM/F-12 containing 5% HS with 100 ng/ml FGF-2 and 5 μg/ml insulin (triangles) provided more than a 10,000-fold increase during passage 4, while CGM (squares) and DMEM/F-12 containing 5% HS with FGF-2 alone (diamonds) or the combination of FGF-2 and IGF-I (circles) also arrived at 1000-fold during passage 4.

the proliferation or differentiation of chondrocytes, including those derived from animals and cell lines in order to widen the screening size. The doses of each soluble factor were those used in previous studies (Table 1).

We confirmed the synergistic effects of FGF-2 with insulin or IGF-I in the human auricular chondrocytes and proved their usefulness in combination with HS. Although it is well known that FGF-2, insulinm and IGF-I are mitogenic to chondrocytes (13,14,30), the mechanisms that promote chondrocyte proliferation seem to be different among such mitogens. Bohme and collaborators reported that insulin or IGF-1 could trigger chondrocyte proliferation under strictly serum-free conditions, although FGF-2 did not promote cell division in serum-free cultures of chondrocytes (3). Quarto and coworkers also described that FGF-2 alone does not induce cell proliferation of chick embryonic chondrocytes without FBS (24). In contrast, FGF-2 could bolster the mitogenic activity of FBS in cultures, indicating that the cells became conditioned by FBS so that they could respond to FGF-2 (3).

This difference in the conditions in which each factor expresses mitogenic activity may be explained by the general concept of competence and progression factors, both of which are required to trigger cell division. The former include FGF-2, EGF, or PDGF, allowing the passage from G_0 to G_1 at the entry in the cell cycle, while the latter correspond to insulin or IGF-I, which commit the cells to DNA synthesis (1). The synergy of FGF-2 with insulin or IGF-I seemed to be associated with the cooperation of these competence and progression factors. In contrast, because insulin and IGF-I, which possess a common role as progression factors, did not compensate each other and could not accelerate the cell cycle effectively, the simultaneous use of both insulin and IGF-I did not help the promotion of proliferation, as seen in the present study. Further studies on signal pathways downstream of FGF-2 and insulin or IGF-I in chondrocytes are anticipated to disclose not only the detailed mechanisms of the synergistic effects of those factors but also to provide the optimal promotion of chondrocyte proliferation.

In contrast, BMP-2 worked rather negatively in the chondrocyte proliferation. In the experiment using the murine chondrocytic cell line ATDC5, BMP-2 upregulated the expression of the type II and type X collagen mRNA and stimulated the sequential progression of the early and late phase differentiation, although it had little effect on the proliferation of the cells (27). Also, for human auricular chondrocytes, BMP-2 is speculated to drive the direction to produce the cartilaginous matrix, but to arrest the progression of the cell cycles.

Regarding the doses of FGF-2, less than 10 ng/ml was used for the promotion of proliferation in chondrocytes and MSC in many previous reports (13,25,28). Quatela and collaborators did not show that doses of FGF-2 greater than 100 ng/ml provide increasing effects on proliferation of human auricular chondrocytes in monolayer culture (25). Because the capacity of a 3D culture system for cells is higher than that in a monolayer culture, even more than 100 ng/ml FGF-2 continues to promote chondrocyte proliferation and maintain the synergistic effects with insulin or IGF-I, as seen in the present data.

After the long-term culture, which provided more than a 1000-fold increase in cell numbers, any kind of media we examined equivalently brought dedifferentiation of the chondrocytes. Chondrocytes embedded in native tissue synthesize type II collagen and proteoglycan, although the same cells cultured in a monolayer lose their typical morphology from round to a spindle fibroblast-like shape and protein synthesis, a phenomenon termed dedifferentiation (29). In order to reverse this inevitable phenomenon, many researchers have used 3D culture embedded in agarose gel (2), alginate beads (4), collagen type I gel (15), or collagen type II gel (16). However, when we applied the 3D embedded culture for chondrocyte proliferation, mRNA expression of the proliferated chondrocytes showed a dedifferentiation

pattern that the Col2a1 expression decreased, compared with those of the primary culture of the chondoryctes. It suggested that dedifferentiation inevitably occurs, even if it may be less in the 3D-embedded culture than in the monolayer culture, when the period of cell culture becomes long. Therefore, when we obtain a sufficient number of cells after a long proliferation culture, we should change the culture system, including the medium contents or cell density, in order to reverse the dedifferentitaion. At present, we have investigated the optimal medium inducing redifferentiation of the chondoryctes, and realized the production of regenerated cartilage possessing mature and abundant cartilaginous matrix in vitro.

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