Bone Regeneration by Transplantation of Human Mesenchymal Stromal Cells in a Rabbit Mandibular Distraction Osteogenesis Model

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Ex vivo expanded mesenchymal stromal cells (MSCs) represent a potential cell population for tissue regeneration strategy. Xenogeneic transplantation using human MSCs (hMSCs) can be an approach to reveal what hMSCs guide in bone regeneration with distinguishable gene expression from a host animal. In this study, we investigated the regenerating effect of hMSCs varying injection time point in a rabbit distraction osteogenesis model. Undifferentiated hMSCs (2×10^6 cells) were injected transcutaneously into the osteotomy site of one side of the mandible 1 day before the onset of distraction (Group 1) or after distraction (Group 2). The contralateral side of the mandible, which was subjected to distraction, but no hMSC injection, was used as the control in each group. hMSCs showed lack of major histocompatibility complex class II expression and suppression of xenogeneic lymphocyte proliferation stimulated by a proinflammatory cytokine. A microcomputed tomography-based evaluation showed a significant increase in new bone volume in the distracted callus in Group 1 compared to the contralateral side. Injection of hMSCs increased the bone mineral density (BMD) of the regenerated bone in both Group 1 and 2, although the former had a higher BMD than the latter. hMSCs of Group 1 subjected to distraction after injection expressed insulin-like growth factor-1 (*IGF-1*) and fibronectin (*FN*), while the expression of most osteoblast differentiation-related markers and growth factors was negligible. These results demonstrated that hMSCs exerted immune suppressive behavior in rabbit T cells *in vitro*, and hMSC transplantation into the distracted callus of a rabbit model provided osteogenic benefits that were more pronounced when the hMSCs were injected just before distraction than at the end of distraction. The beneficial effect of hMSCs might be mediated, partly by the expression of matrix proteins or *IGF-1*, which are known to favor bone formation.

Introduction

BONE MARROW-DERIVED mesenchymal stromal cells (BM-MSCs) have become one of the main cell sources for bone tissue engineering.^{1,2} Various *in vitro* protocols have been developed to induce human mesenchymal stromal cells (hMSCs) to differentiate into mesodermal lineages, such as osteoblasts, chondrocytes, adipocytes, as well as transdifferentiate into tissue cells derived from different germ layers, such as neuronal cells or insulin-producing cells.^{3–5} However, these *in vitro* assay results do not mean that the differentiated cells are functional. It is well known that hMSCs, which undergo osteogenic differentiation express major histocompatibility complex (MHC)-I genes, which leads to their rejection when used xenogeneically.⁶ Thus, immunocompromised animals are required to predict the role of hMSCs in regenerative medicine.^{6,7} However, there are also contradictory argues against probable immunological stimulation induced by

hMSCs in a xenogeneic host system. hMSC transplantation experiment showed that hMSCs maintained their multipotential capacity, and there was long-term engraftment of transplanted cells in a xenogeneic environment.⁸

Despite argues for immunogenicity, several studies have investigated hMSCs in the context of xenogeneic transplantation, because it is difficult to study diverse bone diseases using an immune-deficient animal. In contrary to expectations, there is abundant evidence based on animal studies that *ex vivo* expanded hMSCs improve tissue regeneration and wound healing after *in vivo* transplantation into animals without notable immunological rejection.^{9–11} However, the mechanism by which hMSCs promote tissue regeneration remains unclear, as it is very rare for injected hMSCs to differentiate into cells of the target tissue.^{2,12} Distraction osteogenesis (DO) is a unique model to regenerate bone that involves coupling of mechanical strain and bone cells *in vivo*.¹³ However, the long duration of bone healing can result in a variety of

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complications, such as infection, discomfort, fractures, or mucosal dehiscence.¹⁴ Among the various approaches in place for shortening the consolidation phase of DO, application of autologous or allogenic MSCs has been reported to successfully promote bone growth.^{15–17} The beneficial effects of injected MSCs are explained by the predictable potency of differentiation into active osteoblastic cells based on *in vitro* evidences. Additionally, local transplantation of MSCs was proven to recruit more circulating stem/progenitor cells to the region of injury and contributed to healing.^{17,18} Several mechanisms have been also proposed to explain the virtual role of injected MSCs; namely, secretion of growth factors/cytokines, deposition of extracellular matrix (ECM), and/or adjustment of the immune response and inflammation.^{19–23}

Definitely, either autologous or allogeneic transplantation in a DO model animal is probably a preferable system over xenogeneic transplantation in studying the final effect of MSCs on tissue regeneration.²⁴ For that reasons, there has been not reported so far whether xenogeneic transplantation using hMSCs have an osteogenic effect in a DO model animal. Nevertheless, we approached the xenogeneic setting using hMSCs because we focused to investigate the osteogenic capacity of hMSCs by evaluating not only the bone tissue formation, but also examining the gene expression by hMSCs after transplantation. Specifically, distracted callus is a very active environment, where cell populations respond to a dynamic mechanical strain and are successfully converted to mineralized bone containing well-organized blood vessels.²⁵ Bone cells, including preosteoblasts and MSCs, respond to mechanical stress by altering their rate of proliferation or differentiation status.^{26,27} In particular, increased expression of various cytokines in the local microenvironment surrounding a distracted callus, likely plays a significant role in the communication of mechanical strain to cells.²⁸ With respect to the *in vivo* effect of transplanted hMSCs, we hypothesized that distractive loading followed by cell injection would be effective at inducing hMSCs in a distracted microenvironment to either commit to differentiation into osteoblasts, or cause them to express a diverse set of cytokines to promote bone formation, even in the context of xenogeneic transplantation. Therefore, we investigated the bone regenerating effect of hMSCs injected transcutaneously in a rabbit model in comparison of injection time point between before distraction onset and after distraction, the latter of which is the time point generally used.^{16,17,29} Immune characteristics of hMSCs used in this study were investigated *in vitro* by assessing the expression of the major immunogenic molecule (MHC class I and II), immunomodulatory cytokines (transforming growth factor beta 1 [TGF- β 1] and interleukin [IL]-10), and proliferation of xenogeneic lymphocytes. New bone formation was evaluated by histological and microcomputed tomography (micro-CT)-based analyses. We also explored the gene expression of transplanted hMSCs exposed to distraction by polymerase chain reaction (PCR) using human-specific primers to understand the hMSC role in tissue regeneration.

Materials and Methods

Preparation of hMSCs for transplantation

Commercial hMSCs (PT-2501; Lonza) from the bone marrow of a male aged 36 years were cultured in the specific medium provided according to the manufacturer's instruc-

tion. These cells were verified by the manufacturer to express mesenchymal stem cell surface markers—over 90% of cells were positive for CD105, 166, 29, and 44, and less than 10% were positive for CD 14, 34, and 45. In addition, the hMSCs were also confirmed by the manufacturer to be able to differentiate into adipogenic, chondrogenic, and osteogenic lineages. hMSCs between the third to fifth passages were used for transplantation into the DO model. For injections, a total of 2×10^6 cells (third to fifth passages) were suspended in 0.15 mL physiological saline, and injected directly into the distracted gap of a rabbit mandible.

Fluorescence-activated cell sorting; MHC detection

A total of 2×10^5 hMSCs were cultured with or without interferon (IFN)- γ (100 IU/mL; PeproTech) for 3 days. IFN- γ -treated or nontreated hMSCs were incubated with fluorescein isothiocyanate-conjugated mouse anti-human MHC class I (HLA-ABC) (BD Biosciences), anti-human MHC class II (HLA-DR) (BD Biosciences), or anti-mouse immunoglobulin G (IgG) isotype control for 30 or 90 min in the dark after being washed with a washing buffer (phosphate-buffered saline [PBS] buffer containing 1% bovine serum albumin). Cells were washed twice with the washing buffer and suspended in the washing buffer containing 0.1% paraformaldehyde (Sigma) for fluorescence-activated cell sorting (FACS) analysis. Subsequently, the cells were fixed in 70% alcohol and sorted using the BD FACS Aria Cell Sorting System (BD Biosciences).

Enzyme-linked immunosorbent assay of TGF- β and IL-10

hMSCs were treated with IFN- γ (100 IU/mL) for 3 days, and then were inactivated with 25 μ g/mL mitomycin C. Approximately, 10^4 cells were cultured in a 96-well plate and IL-10 and TGF- β detection were performed after 3 and 6 days. TGF- β /IL-10 levels in the culture supernatants were determined using enzyme-linked immunosorbent assay (ELISA) kits (Quantikine[®]; R&D Systems). Standards for cytokines (0–2000 pg/mL) were run in each series. After incubation, aspiration, and washing, either human TGF- β conjugate (50 μ L/well) or human IL-10 conjugate (100 μ L/well) was added based on the manufacturer's instructions. The OD of each well was determined within 30 min using a microplate reader set to a 450-nm wavelength correction for optical imperfections in the plate. The ELISAs were repeated in triplicate for three or four independent samples ($n = 3–4$).

Mixed lymphocyte cultures

Preparation of peripheral blood mononuclear cells and autologous rabbit MSCs. Peripheral blood mononuclear cells (PBMC) were fractionated from heparinized blood of New Zealand White (NZW) rabbit weighing 3.0–3.5 kg by Ficoll-Paque Plus (1.077 g/mL; Amersham Biosciences) at 300 g for 20 min. The interlayer cells were washed twice with the Hank's buffered salt solution and were ready for lymphocytes proliferation tests. Bone marrow was collected from identical rabbits, which provided PBMC. Heparinized bone marrow was cultured with an expansion medium containing the low-glucose Dulbecco's modified Eagle's medium (Wegene, Inc.), 100 U/mL penicillin, 100 mg/mL streptomycin,

and 10% heat-inactivated fetal bovine serum under a humidified atmosphere of 5% CO₂ at 37°C with medium changes every 3 or 4 days. Adherent cells were expanded for 7 days and were detached by 0.05% (w/v) trypsin/ethylenediaminetetraacetic acid (EDTA). The cells were subsequently passaged two to three times to achieve desired cell numbers. The medium was changed every 3 days during the expansion period.

Proliferation of lymphocytes. Autologous rabbit MSCs (rMSCs) and hMSCs were pretreated with IFN- γ (100 IU/mL) for 3 days, and then exposed to 25 μ g/mL mitomycin C in darkness at 37°C for 20 min, subsequently washed twice, and used as stimulators. Untreated PBMC were to be used as responders. A total of 5×10^4 stimulating rMSCs and hMSCs were cocultured with 8×10^3 responding PBMC in 0.2 mL of lymphocyte culture medium (RPMI 1640 supplemented with 50 μ M 2-mercaptoethanol) for 6 days. The proliferation of responding cells was assessed at days 3 and 6 by using a Cell Counting Kit-8 (Dojindo Laboratories), which employs the tetrazolium salt, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt] according to the manufacturer's protocol.

Surgical procedure for bilateral DO model in rabbit mandible

A total of 28 skeletally mature male NZW rabbits weighing 3.5 kg each were used in this study. All experimental procedures were undertaken in compliance with the guidelines for the Care and Use of Animals described in the guidelines of the local ethics committee of Seoul National University Dental Hospital. All operations were performed under general anesthesia using an intramuscular and intravein injection of Zoletil (0.4 mL/kg; Virbac Laboratories) mixed with Rompun (10 mg/kg; Bayer Korea). After disinfection of mandible with 10% betadine (Potadines; Sam-Il Pharm) and subcutaneous injection of 2% lidocaine containing 1:100,000 epinephrine (Lidocaine HCL Injs.; Yuhan), an incision was along the coronal suture. The inferior border of the mandible was incised

for a length of about 7 cm and the periosteum was elevated under additional local anesthesia to reduce pain. All rabbits underwent osteodistraction of bilateral mandibles. A custom-made titanium external distractor (Hyrex external fixator; Dentaaurum Co.) was adapted by two titanium mini screws (3 mm diameter $\times$ 8 mm length), which were placed across both cortices. A vertical osteotomy was performed on the mandibular body between the first premolar and the mental foramen of the bilateral mandibles by a fissured bur with copious sterile saline irrigation. After the half cut of the mandible, a distractor was fitted surgically on the lateral aspect of the mandible, while the remaining half of the mandible was osteotomized by a fissured bur with copious sterile saline irrigation. After performing the same process to the opposite side, irrigation was done with sufficient normal saline. Suturing was done using 4-0 absorbable nylon (Ailee Co., Ltd.). Postoperatively, all rabbits received antibiotics with intramuscular injection of Cefazolin (55 mg/kg; ChongKunDang Pharm.) for 3 days.

hMSCs transplantation and distraction

The left side of the mandible was then transcutaneously injected with hMSCs on the osteotomized site, while the right side was used as a contralateral control group by injecting 0.15 mL of physiological saline without hMSCs. Rabbits were divided into two different groups based on the time of hMSC injection. Specifically, hMSCs were injected either 1 day before distraction (Group 1), or after distraction (Group 2). After a 7-day latency period, both sides were gradually distracted at a rate of 1 mm/day for 7 days. Gene expression analysis of hMSCs injected into Group 1 was investigated at 4, 8, or 14 days ($n=3-4$ for each time point) after the start of distraction by harvesting a portion of the mandible containing a central distracted callus. Rabbits in Groups 1 and 2 were sacrificed at week 4 of the consolidation phase, and new formation of bone was evaluated for both sides ($n=7$ for each group). Animals were sacrificed at various time points during the DO process, either for analytical bone formation analysis or RNA extraction (Fig. 1).

FIG. 1. Schematic representation of experimental protocols. Rabbits underwent placement of a mandibular distraction device in their bilateral mandibles. This was followed by a 7-day latency period (days -7 to 0), gradual distraction (days 0–7), and consolidation (days 8–35). Day 0 represents the start of distraction. Rabbits were divided into two groups depending on the timing of human mesenchymal stromal cell (hMSC) injection as follows: Group 1 at 1 day before distraction start, and Group 2 at the end of distraction. Gene expression analysis of hMSCs *in vivo* ($n=3-4$, each group) was investigated at 4, 8, or 14 days after distraction was started by harvesting a portion of the mandible containing the central distracted callus from rabbits in Group 1. Rabbits from Group 1 and 2 were sacrificed at week 4 of the consolidation phase and new bone formation was evaluated on both sides ($n=7$, each group). Arrowheads indicate tissue harvest time points for microcomputed tomography (micro-CT) analysis, histology, and/or RNA analysis.

Reverse transcription–polymerase chain reaction/real-time reverse transcription–polymerase chain reaction

For RNA extraction, small pieces of bone fragments containing distracted segments were first cut-out and frozen in liquid nitrogen overnight. The bone samples were then homogenized by crushing in liquid nitrogen, followed by total RNA extraction with 0.5 mL TRIzol reagent (Invitrogen-Life Technologies). Next, 1 µg of RNA from each sample was subjected to cDNA synthesis using SuperScript™ Reverse Transcriptase II (Invitrogen) and oligo (dT)_{12–18} primer (Invitrogen) in a 20-µL reaction volume according to the manufacturer's instructions, and RNA complementary to cDNA was removed using *Escherichia coli* RNase H (Invitrogen). A total of 1 µL of cDNA was then subjected to PCR using the following amplification profile: predenaturation at 94°C for 40 s, amplification (denaturation at 94°C for 40 s; annealing at 60°C for 40 s; extension at 72°C for 1 min) for 30 cycles, followed by a final extension step at 72°C for 10 min. PCR was performed in a DNA thermal cycler (model PTC-200; MJ Research, Inc.). Ten microliters of each PCR reaction mixture was then electrophoresed on a 1.5% agarose gel in the presence of ethidium bromide, and bands were visualized using a Gel Documentation System (Vilber Lourmat). Real-time reverse transcription–polymerase chain reaction (RT-PCR) reactions were performed using SYBR® Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions as follows. Briefly, real-time RT-PCR for the insulin-like growth factor (*IGF-1*) fibronectin (*FN*) and β -actin was carried out in triplicate in three independent experiments ($n=3$). The human-specific primers used in our study were designed using Real-Time PCR System Sequence Detection Software v1.3 (Applied Biosystems), and their sequences are provided in Table 1. For positive and negative controls, cDNA was prepared from cultured hMSCs or bone fragments of normal (noninjected) rabbit mandibles, respectively.

Microcomputed tomography

Animals were sacrificed at 4 weeks postdistracted. Approximately, 2-cm-long mandible segments containing a central distracted gap were extracted and fixed in 10% formalin for 1 week. Micro-CT scans were then taken to quantitatively evaluate new bone formation using the Sky-Scan 1172® Microfocus X-ray System (SkyScan®) with CT software, including CTAn 1.8®, CTvol, and NRecon Reconstruction® (SkyScan). Reconstruction and analyses were performed using NRecon reconstruction and CTAn 1.8 software, respectively. To measure the presence of newly formed bone, a rectangular area of the central position in the distracted callus was selected as the region of interest (ROI) in two-dimensional images, as described previously.³⁰ The pixel zone representing ossification in the defined ROI was then reconstructed in 3D by creating a volume of interest in the lower and upper ranges of the threshold using grayscale units. After using CTAn 1.8 on each reconstructed BMP file, microarchitecture parameters, including the bone volume to the total volume ratio (BV/TV), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp), were obtained using a CT-analyzer in a direct 3D-based analysis of a surface-rendered volume model according to the manufacturer's instructions. To measure bone mineral density (BMD), attenuation data for ROIs were converted into Hounsfield units and expressed as a value of BMD using phantom scans (SkyScan). BMD values were expressed in terms of grams per cubic centimeter of calcium hydroxyapatite (CaHA) in distilled water. A zero value for BMD corresponded to the density of distilled water alone (no additional CaHA), and a value greater than zero corresponded to nonaerated biologic tissue.

Histological and immunohistochemical staining

After micro-CT reconstruction, the mandible fragment containing the distracted area was removed, and then cut in

TABLE 1. HUMAN OLIGONUCLEOTIDE PRIMERS USED FOR REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION AND REAL-TIME REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION

Target gene	GenBank Accession No.	Sequences		Expected size (bp)
		Forward (5'–3')	Reverse (5'–3')	
For RT-PCR				
<i>β-Actin</i>	BC004251	TAT CCA GGC TGT GCT ATC CCT GTA	GAT CTT GAT CTT CAT TGT GCT GGG	583
<i>IGF-1</i>	M 27544	AGA AGC AAT GGG AAA AAT CAG CAG	CAT CTC CAG CCT CCT TAG ATC ACA	328
<i>IGF-2</i>	J03242	GAT GCT GGT GCT TCT CAC CTT CT	GAC TGC TTC CAG GTG TCA TAT TGG	342
<i>TGF-β1</i>	X02812	TGG AAA CCC ACA ACG AAA TCT ATG	CTG AAG CAA TAG TTG GTG TCC AGG	523
<i>VEGF</i>	AF022375	ACC ATG AAC TTT CTG CTG TCT TGG	TAC GTT CGT TTA ACT CAA GCT GCC	550
<i>BMP-2</i>	M22489	CCA TGA AGA ATC TTT GGA AGA ACT	TTA CTA GCA ATG GCC TTA TCT GTG	458
<i>ColI</i>	BC036531	ACC TCA AGA GAA GGC TCA CGA TG	GCT GTT CTT GCA GTG GTA GGT GAT	508
<i>FN</i>	NM_212482.1	GAG CCA TGT GTC TTA CCA TTC ACC	TGG ATA TGG ATA GGT CTG TAA AGG	747
			TTG GCA	
<i>ALP</i>	AH005272	AGC GCA AGA GAC ACT GAA ATA TGC	TGC TTG TAT CTC GGT TTG AAG CTC	699
<i>Cbfa1</i>	NM_004348	CAC CTT GAC CAT AAC CGT CTT CAC	GGT AGG TGT GGT AGT GAG TGG TGG	674
<i>Osteopontin</i>	J04765	CAG GCT GAT TCT GGA AGT TCT GAG	ACT TTT GGG GTC TAC AAC CAG CAT	771
<i>Osteocalcin</i>	X51699	ATG AGA GCC CTC ACA CTC CTC	CTA GAC CGG GCC GTA GAA GCG	302
For real-time RT-PCR				
<i>β-Actin</i>	BC004251	ATT GCC GAC AGG ATG CAG AAG	TTG CTG ATC CAC ATC TGC TGG'	152
<i>IGF-1</i>	M 27544	TGG TGG ATG CTC TTC AGT TCG	TTA GAT CAC AGC TCC GGA AGC	135
<i>FN</i>	NM_212482.1	ACA ACA GCG CCT GAT GCC CC	GTG AGC GGG TGC CAG TG	292

IGF-1, -2, insulin-like growth factor-1, -2; TGF- β 1, transforming growth factor-beta 1; VEGF, vascular endothelial growth factor; BMP-2, bone morphogenic protein-2; Col I, type I collagen; FN, fibronectin; ALP, alkaline phosphatase.

half in the direction parallel to the buccal surface of the mandible. And then, the half cut specimens were decalcified by incubation in a solution of EDTA (7%, pH=7.0) for 10 days, with a solution change every 2 days. The specimens were then dehydrated in 70% ethanol, sectioned longitudinally along the axial plane, and embedded in paraffin by positioning the center portion of bone chip up. After decalcified paraffin sections were cleaned for 10 min with xylene, specimens were cut to 4- μ m thickness without any trimming and stained with Masson's trichrome (MT) to detect bone structures. Digital images of the stained sections were collected using a transmission and polarized light Axioskop microscope, Olympus BX51 (Olympus Corporation). The specimens were then cut into 3–4- μ m-thick pieces (LEICA RM2245; Leica Biosystems Nussloch GmbH), and then either stained with MT or subjected to immunohistochemical (IHC) analysis with the antivascular endothelial growth factor (VEGF), CD31, or osteocalcin antibodies. For IHC staining, paraffin sections were cleaned for 10 min with xylene and the deparaffinized sections were treated with an undiluted serum solution. Specimens were then incubated with anti-VEGF, human anti-CD31, and anti-osteocalcin antibodies (anti-rabbit; Sigma-Aldrich) (all 1:100 dilution), respectively, for 1 h at room temperature, washed twice with PBS, and then incubated with the appropriate secondary antibody, namely, anti-mouse/rabbit IgG-conjugated with horseradish peroxidase, and visualized with a Vectastain kit (Vecta Laboratories) according to the manufacturer's instructions. Section images of stained cells were captured by bright field microscopy.

Labeling with diakylcarbocyanines to trace transplanted hMSCs in vivo

hMSCs were labeled with the fluorescent dye diakylcarbocyanines (Dil; Molecular Probes Inc.) by incubating cells in a Dil dye-containing medium at a final concentration of 10 ng/mL for 24 h. Dil-labeled hMSCs were injected into the distracted gap of the left side of the mandible using the same scheme as described for Groups 1 and 2. The specimens were prepared after sacrifice at 4 weeks postdistraction as per the histological staining protocol described above. Cover slips were mounted in a crystal mounting solution (Biomedica Corp.) and observed under a confocal laser-scanning microscope (Carl Zeiss MicroImaging GmbH).

Statistical analysis

All data are presented as the mean $\pm$ standard error of the mean, and all experiments were performed at least three times. Statistical analyses were performed using SAS 9.1.3 (SAS Institute Inc.). The normality of the data was confirmed by Kolmogorov–Smirnov tests. One-way analysis of variance with the Tukey's *post hoc* multiple comparison test were used to determine significance. Results associated with values of $p < 0.05$ were considered to be statistically significant.

Results

Immunogenicity and immunosuppression assessment of hMSCs

Immune characteristics of commercial hMSCs used in this study were investigated to support that no noticeable im-

munological rejection is associated with the use of hMSCs in rabbits. FACS analysis showed that hMSCs did not express MHC class II (HLA-DR), while about 23% of cells express MHC class I (HLA-ABC) (Fig. 2A). However, over 69% of cells were positive for MHC class I after treatment of proinflammatory cytokine, IFN- γ (100 IU/mL), while the deficiency of MHC class II was maintained at the same condition. Because IL-10 and TGF- β are known to be anti-inflammatory cytokines and regulate immunity, we assessed their secretion by hMSCs (Fig. 2B). IL-10 expression exhibited a minor increase by 23.3% ($p < 0.05$) at day 3 even in the presence of IFN- γ . However, TGF- β expression was not affected by IFN- γ treatment at days 3 and 6. The host lymphocyte response was examined by assessing the proliferation of rabbit PBMCs at day 3 and 6 after coculture either with autologous rMSCs or xenogeneic hMSCs, which were pretreated with IFN- γ or not. The growth of rabbit lymphocytes was inhibited by coculture with IFN- γ -pretreated cells more than nontreated cells. This suppressive effect showed 18.4% ($p < 0.01$) and 13.5% ($p < 0.05$) decrease at days 3 and 6 in coculture with rMSCs, or 26.5% ($p < 0.01$) and 40.6% ($p < 0.01$) decrease at days 3 and 6 in coculture with hMSCs, respectively. Moreover, the proliferation of rabbit PBMCs was lower in coculture with hMSCs, compared to that with rMSCs. Both IFN- γ -pretreated and nontreated hMSCs led to 18% ($p < 0.01$) and 43.6% ($p < 0.01$) decrease of the lymphocyte response, respectively, compared to rMSCs of corresponding condition, which appeared at day 6.

Bone regeneration by hMSCs in the distracted callus of rabbit mandible; micro-CT analysis

We investigated bone regeneration by hMSC transplantation in a DO model for two different injection time points. In Group 1 rabbits, hMSCs were injected transcutaneously into the osteotomized site just 1 day before the start of distraction, whereas in Group 2 rabbits, hMSCs were administered after distraction. The time-frame used in Group 2 has been used in previous studies,^{16,17} while the timing of hMSC injection that we used in Group 1 is a novel approach. New bone structures of the distracted callus were evaluated using micro-CT analysis, which calculates morphometric parameters from a selected ROI (Fig. 3). BV/TV indicates the fraction of mineralized tissue, while Tb.Th and Tb.Sp provide detailed information concerning the thickness and organization of trabeculae, respectively. These parameters of micro-CT analysis revealed that the BV/TV of the contralateral side in each group showed around 25% of the bone regeneration of the distracted callus with the same ratio as BV. hMSC transplantation led to an increase in BV/TV in Group 1 by 32.6% and Group 2 by 28.7%. The BV/TV of Group 1 was significantly greater (24%; $p < 0.05$) for the hMSC-transplanted side than the contralateral side, while the increase in BV/TV in Group 2 was not significant. hMSC transplantation in Group 1 led to a BV/TV that was 18.5% higher compared with Group 2, which is a significant increase. However, the values of the other parameters, namely, Tb.Th and Tb.Sp, were similar within and between groups. All parameters of the microstructures in Group 1 were similar to those of Group 2 except for BMD. Indeed, hMSC transplantation increased BMD in Group 1 significantly by 63.3% ($p < 0.01$) and in Group 2 by 95.3% ($p < 0.01$) compared to the respective control sides. Group 2 had a BMD value of 0.707 g/cm³ for

FIG. 2. Immunogenicity of hMSCs. **(A)** Expression of major histocompatibility complex (MHC) class I (HLA-ABC) and II (HLA-DR) by flow cytometry in hMSCs. Plots show MSC before (left) and after (right) induction with interferon (IFN)- γ (100 IU/mL) for 3 days. Dotted lines indicate isotype-matched mouse immunoglobulin G negative control. Solid curve is fluorescence result after staining with Ag-specific Abs. **(B)** Enzyme-linked immunosorbent assay (ELISA) of transforming growth factor (TGF)- β and interleukin (IL)-10 from hMSCs. hMSCs were treated with IFN- γ (100 IU/mL) for 3 days, then were inactivated with mitomycin. Extracellular release of TGF- β and IL-10 was quantified in cell culture supernatants ($n > 4$) at days 3 and 6 using an ELISA kit. hMSCs displayed increased secretion of IL-10 at days 3 in the IFN- γ -treated group, while there was no difference between IFN- γ -treated and -untreated groups at days 6. TGF- β secretion was not unchanged after IFN- γ treatment at days 3 and 6. Significantly different from the control, ** $p < 0.01$. **(C)** Mixed culture of rabbit lymphocytes. Autologous rabbit MSCs (rMSCs) and hMSCs were treated with IFN- γ (100 IU/mL) for 3 days, then were inactivated and cocultured with rabbit peripheral blood mononuclear cells. Both autologous rMSCs and hMSCs displayed a significant inhibition on lymphocyte proliferation after IFN- γ treatment. This inhibitory effect of hMSCs on the lymphocyte response was stronger compared with autologous rMSCs. Significantly different from the control, * $p < 0.05$, ** $p < 0.01$.

FIG. 3. Micro-CT analysis; effect of hMSCs on new bone formation in a rabbit mandible distraction osteogenesis (DO) model. New bone structure in the distracted callus was evaluated using micro-CT analysis at 4 weeks after distraction was started in Groups 1 and 2. **(A)** bone volume/total volume (BV/TV) (%), **(B)** trabecular thickness (Tb.Th) (mm), **(C)** trabecular separation (Tb.Sp.) (mm), and **(D)** bone mineral density (BMD) (g/cm³) of newly ossified tissue are expressed as the mean $\pm$ standard deviation in hMSC-injected and contralateral controls. Significant differences between groups: * $p < 0.05$, ** $p < 0.01$. **(E)** Three-dimensional images from micro-CT in contralateral and hMSC-injected side of Group 1 and 2 were presented at week 4 of the consolidation period. hMSC-transplanted callus of each group revealed more densely mineralized structure than contralateral side.

the hMSC-transplanted side, which is close to that of normal rabbit bone, and this value was 42.5% higher than the BMD of the contralateral side ($p < 0.01$). Micro-CT reconstruction of a rectangular area of the central position in the distracted callus showed more dense bone formation in hMSCs injected side than the the contralateral side both in Groups 1 and 2 (Fig. 3E).

Histological examination

Histological observations of MT-stained sections revealed no inflammatory reactions in and around the regions where new bone had formed (Fig. 4A). After 4 weeks of consolidation, the central zone in the defect space of the hMSC-

transplanted side was filled and bridged with new bone, and a well-formed calcified inorganic bone matrix was present. The contralateral side of each group had a relatively loose bone structure appearance. There was little difference in the hMSC-transplanted side between Groups 1 and 2. The new bone region of Group 1 was further investigated by IHC staining for osteocalcin (a late osteoblast differentiation marker), VEGF-A, and CD31. The osteocalcin antibody staining pattern correlated well with the histological results of MT staining, indicating that hMSC transplantation enhanced osteocalcin expression by recipient cells (Fig. 4B). We also examined the expressions of proteins related to angiogenesis, including VEGF-A, which is one of several VEGF

FIG. 4. Histological appearance of newly ossified callus after hMSC transplantation into a rabbit mandible DO model. **(A)** Histological sections of newly ossified callus in Group 1 and 2 were stained with Masson's trichrome 4 weeks after distraction was started in the same experiment described in Figure 3 (original magnification, 12.5 $\times$ and 100 $\times$). The bars represent 1000 μ m and 100 μ m in 12.5 $\times$ and 100 $\times$ magnification, respectively. **(B)** Immunohistochemical (IHC) staining for osteocalcin, vascular endothelial growth factor (VEGF), and CD31 expression was performed at the same time point as in **(A)** (original magnification, 200 $\times$). *Indicates stained region in each IHC staining. The bars represent 100 μ m. Broken lines indicate the distracted callus region. Color images available online at www.liebertpub.com/tea

types and plays a specific role in controlling the growth of new blood vessels by mediating endothelial cell proliferation, migration, and organization into functional vessels.^{31,32} However, VEGF expression does not confirm a human-specific response, because the anti-VEGF-A antibody that we used has cross-reactivity with mouse/rabbit and human cells. In addition, we analyzed expression of CD31, a specific surface marker of endothelial cells,³³ using a human specific anti-CD31 monoclonal antibody that has been reported not to cross-react with mouse/rabbit endothelial cells.³⁴ VEGF expression was more apparent in the hMSC-transplanted side compared to the contralateral side. Further, IHC staining with the human anti-CD31MAb resulted in positively stained spots around blood vessel-like structures on the side transplanted with hMSCs, but not on the contralateral side.

To identify hMSCs after *in vivo* transplantation, Dil-labeled hMSCs were traced after a 4-week healing period in both Groups 2 and 3 (Fig. 5). A very small fraction of Dil-labeled hMSCs were present at this time point, located mainly near the surface of the new bone area or bone marrow. However, it was unclear whether the detected hMSCs were present as undifferentiated stromal cells, osteoblasts, or some other kind of cell.

Gene expression of transplanted hMSCs exposed to distraction

We investigated the gene expression pattern of hMSCs transplanted into Group 1 during the distraction phase when

these transplanted hMSCs are exposed to a dynamic tissue environment with distractive loading. RT-PCR was performed using bone specimens obtained from Group 1 at 4, 8, and 14 days after distraction (Fig. 6). Cultured hMSCs used as positive controls generated fragments of the expected size that corresponded to the transcripts for most of the analyzed genes. The primer for β -actin was designed to be human sequence-specific, but there was minor expression in the negative control derived from rabbit mandible, indicating a nucleotide sequence similarity between the rabbit and human β -actin genes. RT-PCR revealed a lower expression of β -actin on the contralateral than the hMSC-transplanted side, indicating that hMSCs probably migrated from the injected side into the contralateral side. Furthermore, β -actin expression declined at both the injected and contralateral side as time passed. Fragments of only three genes—*IGF-1*, fibronectin (*FN*), and type I collagen (*Col I*)—were successfully amplified from hMSCs. Osteogenesis-related growth factors (*IGF-2*, *TGF- β 1*, *VEGF*, and bone morphogenic protein-2 [*BMP-2*]) and osteoblast differentiation-related markers (*alkaline phosphatase*, *osteopontin*, *osteocalcin*, and *osteopontin*) could not be amplified. *Col I* expression was transient with no increase in expression, 4 days after the start of distraction.

We performed statistical analysis of the real-time RT-PCR results for β -actin expression (Fig. 7). Human-specific gene expression was analyzed by comparing the cycle number of each gene amplification with that of a blank control, which was a real-time RT-PCR reaction mix without cDNA. Human-specific β -actin amplification from cDNA extracted

FIG. 5. Tracking transplanted hMSCs *in vivo*. hMSCs were labeled with fluorescent diacylcarbocyanines (Dil) dye (10 ng/mL) and injected into the distracted gap of the left side according to the same scheme used for Groups 1 and 2. Newly ossified callus in Group 1 and 2 were prepared after sacrifice, 4 weeks after distraction. Images at left end, middle position, and right end are optical (A, D, G), fluorescence (B, E, H), and merged (C, F, I) photomicrographs, respectively. hMSCs appeared near the surface of new bone area or bone marrow. *Indicate remained Dil-labeled hMSCs *in vivo*. The bars represent 20 μ m. Color images available online at www.liebertpub.com/tea

FIG. 6. Images of reverse transcription–polymerase chain reaction (RT-PCR) for (A) osteogenesis-related growth factors (*IGF-1*, *IGF-2*, *TGF- β 1*, *VEGF*, *BMP-2*), (B) osteoblast differentiation-related markers (*alkaline phosphatase* [*ALP*], *Cbfa I*, *osteopontin*, *osteocalcin*, *Coll*, and *FN*) in hMSC-injected side of Group 1. Rabbits were sacrificed at 4, 8, and 14 days after distraction was started and total RNA was extracted from small pieces of bone fragments containing distracted segments. Positive control (PC) indicates RT-PCR using cultured hMSCs, while the negative control (NC) indicates RT-PCR using rabbit bone from the mandibular fragment.

from the contralateral side on days 4 and 8 occurred around the 27.8th cycle and 30.5th cycle, respectively. In the blank sample, amplification occurred at the 28.9th cycle (data not shown), indicating negligible human gene expression on the contralateral side. In contrast, human β -actin amplification occurred around the 26.6th cycle for cDNA extracted at day 4 and the 27.1th cycle for cDNA extracted at day 8 from the hMSC-transplanted side. Indeed, quantitative calculation of β -actin expression revealed that expression in the hMSC-treated side was 3.6-fold higher than that on the contralateral side on day 4. However, human β -actin expression in the hMSC-injected side decreased to 37.7% at day 8 compared to that at day 4, suggesting that the population of hMSCs declined with time after transplantation. Fold differences in the levels of each gene were calculated for each group without the step of normalizing C_T values using the housekeeping gene β -actin, because the expression of human-specific β -actin was different on both sides and declined over time. Similar to the expression of the human-specific β -actin gene, *IGF-1* and *FN* expression in the contralateral side was negligible and declined as time passed. In comparison with the expression at day 4 on the contralateral side, *IGF-1* and *FN* expression in the hMSC-injected side was around 107-fold and 7-fold, higher, respectively. The fold change of gene expression at day 4 in the hMSC-injected side decreased to 40.8% for *IGF-1* and 25.3% for the *FN* gene at day 8.

Discussion

MSC administration has been regarded as an alternative to try to reduce the long consolidation period associated with DO and to overcome possible fibrous union or nonunion.^{16,35} This effect of *ex vivo*-expanded MSCs has been investigated through autologous or allogeneic transplantation, typically performed after distraction. We performed xenogeneic transplantation with BM-hMSCs and investigated the effects of injecting hMSCs before distraction onset. Our major findings are that hMSCs exhibited an immune-suppressive

FIG. 7. *In vivo* gene expression profile of transplanted hMSCs in Group 1. Rabbits were sacrificed at 4 and 8 days after distraction was started and total RNA was extracted from small pieces of bone fragments containing distracted segments both in contralateral side and hMSC-injected side. Human-specific β -actin (A), *IGF-1* (B), and *FN* (C) expression were analyzed by real-time RT-PCR and RT-PCR using day 4 and day 8 specimens. Significant differences between day 4 and day 8 specimens: * p < 0.05, ** p < 0.01.

effect on rabbit lymphocytes *in vitro*, and hMSC injection enhanced bone regeneration in a xenogeneic rabbit DO model more efficiently at time point of predistracted than postdistracted. We also found that the injected hMSCs expressed only a limited subset of osteoblast differentiation-related genes, including *FN* and *IGF-1*, which may be one of the mechanisms by which these cells contribute to bone regeneration in xenogeneic recipients.

The immune suppressive effect of hMSCs has been very often reported in allogenic or xenogenic settings *in vitro* or *in vivo*.³⁶ Immunogenicity of hMSCs are characterized to be negative for immunologically relevant surface markers, which include MHC-II, CD40, CD34, CD80, and CD86³⁷ and display an inhibitory effect on the proliferation of allogenic T cells *in vitro*.^{38,39} Our immunophenotypic analysis of hMSCs revealed that undifferentiated hMSCs express MHC class I, but not class II. Addition of IFN- γ for 3 days induced greater than 69% of cells to express MHC class I, as like in other literatures.^{6,36,37} To date, it is widely accepted that MHC class I is expressed on virtually all nucleated cells. MSCs expressing MHC class I are reported that they are not recognized by the immune system.³⁷ Interestingly, MHC class II expression of hMSCs in the current study kept missing even in the presence of IFN- γ . This property was different with the results shown in the aforementioned literatures that the negative expression of MHC class II was changed to be up-regulated after exposure to IFN- γ ,^{6,36,37} probably due to individual specificity or difference of culture method. Another property of immunogenicity of hMSCs revealed that the immunosuppressive effect of hMSCs on the proliferation of xenogenic rabbit lymphocytes was more efficient compared with autologous rMSCs. Moreover, this suppression on the lymphocyte response was more noticeable with IFN- γ -pretreated hMSCs than nontreated cells. These data indicate that undifferentiated hMSCs used in this study are immunosuppressive and modulate immune responses in the rabbit host system, supporting that hMSCs can be used in the xenogeneic animal model for tissue engineering purposes.

The basis for a xenogenic setting is that it provides an alternative in the study of regeneration potential of hMSCs because of the limitation of the allogenic human model for ethical reasons. It is very intriguing that xenogeneic transplantation of hMSCs significantly promoted new bone formation of distracting callus in the current study. There are still active argues for the osteogenic potential of hMSCs in a xenogeneic setting even though hMSCs prepared from BM have a greater potential to differentiate into osteoblasts compared with tissue from other sources.⁴⁰ A skeptical perspective comes from the evidence reporting that the xenogenic transplantation of hMSCs coupled with scaffolds via loading on collagen sponge or hydroxyapatite to poorer bone regeneration than autologous transplantation in the rabbit or ovine defect model.^{24,41} Furthermore, some studies have indicated that allogeneic and xenogeneic BM-MSCs are likely to be rejected when induced to differentiate into osteogenic cells based on their expression of histocompatibility antigens.^{6,7} Therefore, it was thought that for skeletal regeneration, MSCs have to be autologous to avoid the need for immunosuppressive drugs. On the contrary, there is also abundant evidence based on animal studies that *ex vivo*-expanded hMSCs improve injury repair in a range of tissue, including bone or neuron, after *in vivo* transplantation into

animals without any notable inflammation.^{9–11} Consistently, our previous studies have shown enhanced bone regeneration in a rat calvarial defect model using BM-hMSCs delivered by a hydrogel carrier.^{10,11} There is no doubt that for direct orthotopic applications, it would be helpful to use appropriate scaffolds to hold cells in place and support their differentiation.⁷ However, in DO studies, MSCs have been delivered using direct injection either with an injectable gel¹⁵ or recently by suspension in a saline solution^{17,29} into the osteotomy site, probably due to the anatomical and pathological characteristics of DO. Therefore, based on our expectation that hMSCs would enhance bone regeneration in a rabbit DO model, we performed direct injection of hMSCs without a carrier, and exposed the hMSCs to mechanical stimulation after transplantation. Moreover, we used a bilateral rabbit mandible model to study the *in vivo* osteogenic effect of hMSCs, which is not possible in rodents because of their small jaws. Our results indicate that xenogenic transplantation models can be used to investigate the osteogenic potentials of hMSCs coupled with growth factors or mechanical stimulation.

Another interesting finding is that transcutaneous injection of hMSCs before distraction improved bone formation compared to hMSCs injection after distraction. MSCs have generally been injected at the end of distraction^{16,17,35} because it was previously reported that the timing of injection has no effect on experimental outcomes in DO.¹⁵ Moreover, postdistracted timing appears to be more suitable in terms of the availability of spaces for cells to anchor to the bone. We found that the BMD of regenerated bone was significantly higher after hMSC injection regardless of timing, while hMSC administration did not appear to affect Tb.Th and Tb. Sp in comparison with regular DO without any treatment. However, hMSC injection before distraction led to higher BV/TV and BMD values than hMSC injection after distraction. Moreover, the BMD value of the group that received an hMSC injection before distraction was close to the BMD value of the normal mandible. The positive effects of hMSC transplantation suggests that administration of *ex vivo*-expanded BM-hMSCs could potentially accelerate bone regeneration in distraction gaps, and transcutaneous injection before distraction might be considerable if applied in DO therapy.

Despite the availability of a large amount of data regarding the multilineage differentiation potential of MSCs *in vitro*,⁴² transplanted MSCs rarely differentiate into the cell type that constitutes the injured tissue itself.^{43,44} Indeed, we also observed this in our current investigation. The xenogenic setting using hMSCs in the current study is to trace the gene expression of administered MSCs in the host system, and then reveal the role for bone regenerating effect as well. Otherwise, there is a rare method to distinguish the gene expression of administered MSCs from that of recipient cells if autogeneously or allogeneously transplanted. Specifically, after transplantation, hMSCs were subjected to the same distractive loading that is known to induce osteoblast differentiation of resident cells in the same local microenvironment.⁴⁵ Nevertheless, the transplanted hMSCs exhibited no signs of differentiation into tissue-specific cells, and they did not express osteoblast differentiation-related markers during either the distraction or consolidation phases, despite intrinsic expression of these same genes *in vitro*. Immune

rejection by the host may limit the osteoblastic differentiation of hMSCs, or the physiological *in vivo* signals may not have been sufficient to drive donor hMSCs into an osteoblastic lineage, in contrast to our expectations. However, there might be a possibility that extremely minor portions of transplanted hMSCs would be differentiated into osteoblasts or endothelial cells despite of no signs for differentiation in RT-PCR. Moreover, the positive influence of the hMSCs injection despite the lack of osteogenic-related differentiation markers might be explained by the suggestions, which have been proposed by researchers, including secretion of growth factors/cytokines, deposition of ECM, and/or adjustment of the immune response and inflammation.^{19–23}

The survival rate of MSCs after transplantation is still a topical issue in MSC-based therapy, because the number of MSCs declines drastically with time. We confirmed this phenomenon; β -actin expression by hMSCs decreased in a time-dependent manner, which may be due to the apoptosis of transplanted hMSCs or the migration of hMSCs from the injected site into other regions. However, the latter argument is not convincing, because a previous study reported a high likelihood of recruitment of hMSCs to the distracted callus because the secretion of SDF-1, an MSC attractant, is significantly increased during the distraction period in patients undergoing DO.³⁴ We found active expression of *IGF-1* and *FN* in xenogeneic recipients, although the results of the present study do not offer any clues as to why the other genes that we evaluated were silenced after transplantation. *IGF-1* and the basic fibroblast growth factor (bFGF) are induced during distraction in various cells located in the distracting area, in addition to BMP-2, BMP-4, and/or TGF- β 1.^{28,46,47} *IGF-1* and bFGF in the distracted region are thought to be responsible for osteoblast proliferation and osteoblast differentiation from precursor mesenchymal cells, leading to *de novo* bone formation.⁴⁶ Indeed, exogenous *IGF-1* has been shown to significantly enhance osteoblastic activity during distraction, resulting in bony unions.⁴⁸ Nevertheless, there is no doubt that transplanted MSCs exert a positive influence on osteoblastic activity through paracrine signaling of *IGF-1* expression during the distraction phase. *FN*, as one of the classes of ECM, is an adhesive protein that can act as a biological glue to mediate interactions between cells and other ECM proteins, thereby creating a provisional matrix that promotes cell migration and adhesion.⁴⁹ Moreover, interactions among the ECM, growth factors, and cells underlie the processes of tissue generation and regeneration.⁵⁰ There is sufficient evidence that trophic molecules, such as ECM molecules, cytokines, and growth factors mediate a number of effects at the sites of tissue injury that stimulate tissue regeneration.^{42,51–53} Therefore, it is possible that the expression of *FN* and *IGF-1* from transplanted hMSCs might have facilitated a pro-osteogenic microenvironment in the distracted callus, leading to the observed improvement in BMD, although the expression of both genes declined as time passed, similar to the pattern seen for β -actin expression.

Angiogenesis is a prerequisite for mineralization to begin in DO.⁵⁴ In the present study, new bone regions injected with hMSCs are apparently in increased expression of the angiogenic cytokine VEGF; however, expression of human-specific VEGF was not detected by RT-PCR, indicating that hMSCs may have induced VEGF release via the recruitment of resident cells through an undefined trophic effect. As some

studies have indicated, the actual number of MSCs that successfully differentiate into vascular structures is quite low,¹⁸ and further, the differentiation potential of hMSCs administered into vascular endothelial cell lineages is very limited. Nevertheless, in a previous study, calluses regenerated with transplanted hMSCs stained positive with an antibody specific for human CD31, which is used as a specific agent to detect human endothelial cell-lined blood vessels in live animals.⁵⁵ Our results suggest that a few of the hMSCs injected into a distracting callus were able to differentiate into vascular cells or secrete signals capable of recruiting endothelial progenitor cells from the circulating blood. Another possibility is that given the pericyte nature of BM-MSCs, the hMSCs associated with vascular cells, thereby stabilizing them during blood vessel ingrowth into the diseased or injured tissue.⁵⁶ The findings in the current study are consistent with the results we obtained previously when we injected hydrogel-mixed hMSCs into a rat calvaria defect model.¹⁰

In conclusion, we investigated the osteogenic effects of xenogeneic transplantation of BM-hMSCs into a rabbit mandibular DO model. Transcutaneous injection of hMSCs without carrier into the osteotomy site increased BV/TV and the BMD of the regenerated callus, and these effects were more pronounced when hMSCs were injected just before distraction rather than at the end of distraction. The transplanted hMSCs only expressed a couple of genes and the expression of these genes decreased as time passed. Used hMSCs are characterized to be negative or express less for histocompatibility antigens and display an inhibitory effect on the proliferation of xenogenic rabbit lymphocytes *in vitro* even in the treatment of proinflammatory cytokine. Nevertheless, the osteoblastic differentiation potential of transplanted hMSCs was very limited in the xenogeneic host system. Instead, hMSCs expressed *IGF-1* and *FN* expression in the xenogeneic host system, which may play an important role for the observed increase in bone regeneration even though it appeared only in an initial short period after hMSC transplantation. Our current results suggest that xenogeneic transplantation of hMSCs can be regarded as a feasible experimental model for diverse osteogenic studies.

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Disclosure Statement

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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