1. Introduction

The concept of tissue engineering, that is, seeding cells on a scaffold and inducing tissue formation in the presence of growth factors, was proposed by Professor Joseph Vacanti of Harvard University and Professor Robert Langer of the Massachusetts Institute of Technology in 1993 [2]. The photograph of a human ear mounted on the back of a mouse they presented was covered widely by the mass media throughout the world, gaining immediate fame for regenerative medicine. Thereafter, research in the field of regenerative medicine has advanced greatly. Two years ago, Professor Shinya Yamanaka of Kyoto University was awarded the Nobel Prize, and induced pluripotent stem cells (iPS) cells and regenerative medicine have also become hot topics in Japan.

In the maxillofacial region, nearly all disorders have become research subjects of regenerative medicine, including loss of teeth and periodontal tissue due to caries and periodontal disease, hypoplasia or defects of bone and cartilage associated with congenital anomalies, such as cleft lip and palate, defects of bone, cartilage, and soft tissues due to tumors or trauma, and xerostomia. However, the dental field has a long history of knowledge of materials, and treatment using biological materials has been established such as crown prostheses, dentures, dental implants, and epitheses. In addition, with the recent advancements in medical technology, reconstruction of large mandibular defects has become possible by microsurgical operations. Therefore, to surpass such a conventional approach as an alternative, regenerative medicine should take an approach of being less invasive and/or more effective. Particularly, the application of regenerative medicine is considered to be advantageous for the maxillofacial region because the weight load is smaller than in the limb, and the amount of tissue needed for reconstruction is generally small. In this report, we present our preclinical and clinical research on bone and cartilage regenerative medicine in the oral and maxillofacial region.

2. In situ tissue engineering in maxillofacial region

After tumor resection or trauma in the maxillofacial region, bone defects occasionally impair the function of mastication and the facial appearance of patients, worsening their quality of life (QOL). To reconstruct such defects, many surgical procedures have been developed including block bone graft and vascularized bone graft. Especially in mandibular reconstruction, a vascularized free graft of the fibula is preferably chosen because: (1) a sufficiently long bone with vessels can be harvested, (2) it is thick enough for placing dental implants, (3) the bone graft can be harvested during manipulation in the head region, (4) skin grafting can be additionally performed, and (5) complications of the donor site are rare. However, this operation requires intraoperative trimming of the bone graft to adjust its shape to the recipient site, making it difficult to provide satisfactory morphological form. In addition, it is highly invasive at the donor site, and there are limitations in the size and shape of the grafts.

For these reasons, particulate cancellous bone and marrow (PCBM) from the ilium is often transplanted in combination with a titanium mesh tray (Fig. 1) [3]. By appropriate shaping of the tray, this method enables more morphologically natural reconstruction, while restoration of adequate occlusion is also possible with the use of dentures and dental implants. Moreover, mesenchymal cells in PCBM can induce new bone formation and subsequent bone resorption, resulting in bone remodeling that matches the surrounding recipient bone. This is a peculiar property of PCBM, in contrast to the aim of a conventional block bone or vascularized bone graft, which is the survival of the graft bones itself. Due to this osteogenic ability of PCBM, reconstruction using PCBM can be regarded as in situ tissue engineering (it can also be considered "in vivo tissue engineering," because tissue regeneration proceeds after transplantation) [4]. Another example based on in situ tissue engineering in the maxillofacial region is bone lengthening for micrognathia, in which bone formation can be induced by mild traction on the callus of an osteotomy site. It is therefore considered that regenerative medicine may not be such a new approach, as some conventional treatments adopt the concepts of tissue engineering.

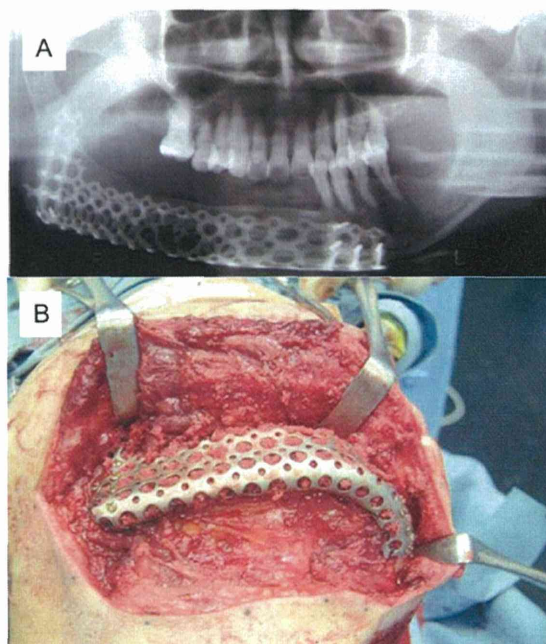

Fig. 1. Bone reconstruction using particulate cancellous bone and marrow (PCBM) in combination with a titanium mesh tray (excerpted from Ref. [3] with permission). (A) A case of reconstruction using PCBM and a titanium mesh tray. Nine-month postoperative panoramic view showing the successfully reconstructed mandible. The occlusal function of this patient is quite good with her remaining natural dentition and removable partial denture. (B) Intraoperative view after PCBM grafting. PCBM was loaded into the mesh and densely condensed as much as possible.

3. Preclinical research on bone regenerative medicine

Vascularized bone and block bone are considered to retain the intrinsic properties of bone, because they contain the original cell components and growth factors accumulated in the matrix. Therefore, bone union after grafting of these bones usually progresses smoothly, resulting in a favorable clinical outcome. Transplantation of PCBM with a titanium mesh tray also exhibits excellent results. However, as long as autologous transplantation is used, these operations include the process of harvesting tissues, and the amount obtained for grafts may be limited.

In foreign countries, particularly in the United States, use of allogeneic bone is prevalent. The amount and shape of bone grafts can be managed with allogeneic grafting, and the operation is less invasive with respect to the donor site. While cells in allogeneic bone are not viable and the activity of growth factors in the matrix is suppressed by freezing, the grafts are still natural bone with excellent function. However, the use of allogeneic bone has not been prevalent in Japan because of concerns of infection and ethical issues. Instead, calcium phosphate-based artificial bone has been used as an alternative [5]. The reason why calcium phosphate is preferred is that being a component of bone, it has an excellent biocompatibility and biological safety. Besides, the supply of calcium phosphate is unlimited as it is synthesized from limestone and phosphate rock.

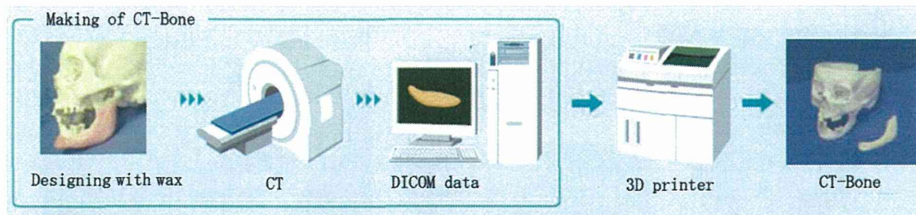

Fig. 2. Fabrication of custom-made artificial bone (CT-Bone). The design of CT-Bone was shaped on a 3D plaster figure of the patient with wax, which was then converted to DICOM data by CT imaging. Then, CT-Bone was printed using laminating tricalcium phosphate powder with a 3D ink-jet printer.

In the application of artificial bone, calcium phosphate is usually manufactured into porous blocks, granules, or paste. As granules and paste cannot retain a given shape, they are mostly used to fill defects. Porous blocks can retain their shape, but require trimming during operation, failing in manipulability and precision. Overall, artificial bone remains inferior to autogenous or allogeneic bone in terms of strength, morphological form, manipulability, degradability, resorbability, and osteogenic ability.

To that end, we, together with Professor Ung-il Chung of the University of Tokyo and Graduate Schools of Engineering and Medicine, have developed artificial bone that would maximize bone formation, but would be subsequently replaced by autologous bone. We used computed tomography (CT) image to make custom-made artificial bone (CT-Bone) that would fit the defect of each patient (Fig. 2). Regarding the procedure, we designed the shape of CT-Bone on a three-dimensional (3D) plaster figure of the patient using wax, which was then converted to Digital Imaging and Communications in Medicine (DICOM) data after CT imaging. Based on the data, CT-Bone was fabricated with a 3D ink-jet printer [6]. During 3D printing, a thin layer (0.1 mm) of tricalcium phosphate (TCP) powder was first made, and then a hardening liquid was sprayed over the powder. By repeating the process, not only the external shape but also the internal structure of artificial bone can be fabricated.

4. Clinical application of custom-made artificial bone

After performing preclinical studies using large experimental animals, from March to July 2006 we conducted clinical research on CT-Bone in 10 patients with maxillofacial deformities in non-weight-bearing regions because of congenital malformation, trauma, or tumor resection. Subsequently, a clinical trial was conducted in another 20 patients from October 2008 to September 2009 (Figs. 3 and 4) [7,8]. As CT-Bone was prepared from the patients' CT images, it fitted the recipient sites well, and did not require adjustment of the shape during operations. In addition, the grafts were strong enough to tolerate the impact of the operation (20 MPa) without sintering after modeling, showing excellent manipulability. Thus far, the outcomes have been non-problematic in terms of safety, and union between CT-Bone and self-bone at the recipient site has been confirmed. However, we need to bear in mind that CT-Bone is still a foreign material at the time of transplantation, and thus should not be transplanted at irradiated or infected sites.

To broaden the application of CT-Bone to weight-bearing regions, we are now developing highly functional artificial bone by hybridization with other materials including metals and fusion with bone-inducing signals. As CT-Bone does not have sufficient mechanical properties for the reconstruction of segmentally resected mandible, we are now developing another approach for bone regeneration in such cases.

5. Research background of cartilage regenerative medicine

Cartilage is present in not only joints and intervertebral discs but also the nose, ear, and trachea in the head and neck region. It plays an important role in maintaining morphological form and activities of daily life. Cartilage is generally deficient in self-repair ability, and once it is damaged, spontaneous restoration is unlikely to occur. Therefore, trauma such as injury of articular cartilage, age-associated disorders such as osteoarthritis, and inflammatory

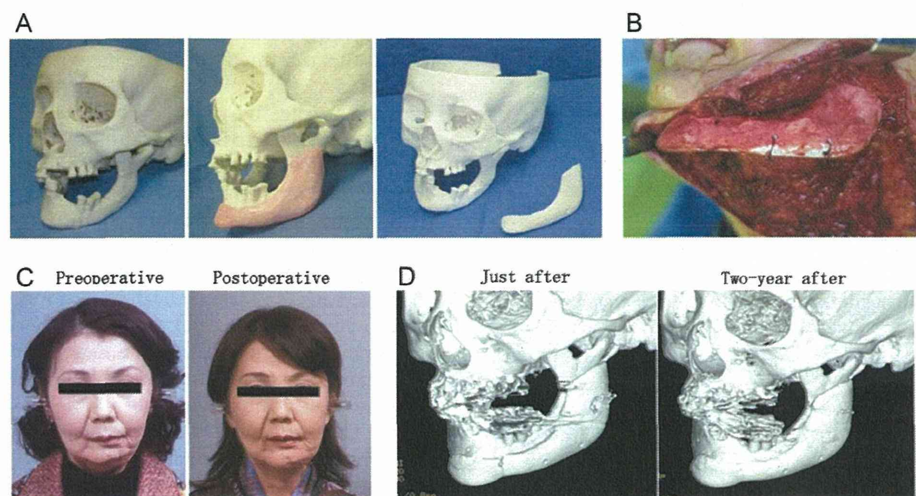

Fig. 3. Clinical application of CT-Bone: Case 1 (excerpted from Ref. [7] with permission). (A) On a 3D plaster model of the patient (Left), the design of CT-Bone was shaped using wax (Middle), which was then converted to DICOM data after CT imaging. CT-Bone was fabricated by an ink-jet printer (Right). (B) Transplanted CT-Bone during operation. (C) Preoperative and postoperative photographs of patient's face. (D) CT images just after and 2-years after the operation. Union between CT-Bone and self-bone at the recipient site has been confirmed.

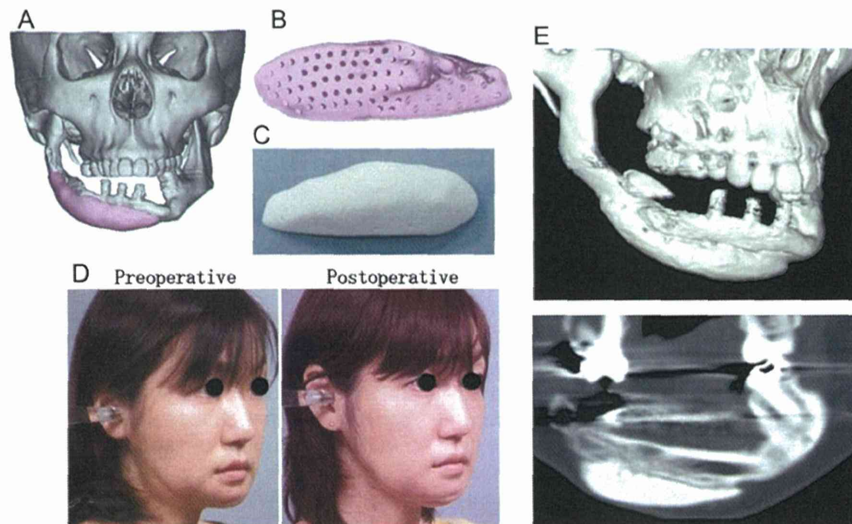

Fig. 4. Clinical application of CT-Bone: Case 2 (excerpted from Ref. [8] with permission). (A) CT image of plaster model with the structure of CT-Bone. (B) Structural design of CT-Bone, which has pores on the bone interface. (C) CT-Bone fabricated by 3D printing. (D) Preoperative and postoperative photographs of patient's face. (E) One-year postoperative CT images.

disorders such as rheumatoid arthritis often impair the activities of patients. Conventional treatment by joint replacement or autologous cartilage grafting has problems, such as poor durability, infection, and invasiveness to the donor site.

In the field of regenerative medicine, cartilage regenerative medicine is relatively advanced, along with regeneration of skin and cornea. Autologous chondrocyte implantation (ACI) has been widely introduced, particularly in Europe and the United States [9]. In the original method of ACI for local defects in articular cartilage, chondrocytes, isolated from articular cartilage of non-weight-bearing regions, are proliferated by culture, administered into the defects as a cell suspension, and the donor site is covered with a periosteal patch to prevent leakage (Fig. 5). However, the indications for ACI are still limited, and complications such as laminar detachment, thickening of the periosteal patch, and graft displacement have been reported [10]. In addition, it has been suggested that there may not be a clear clinical advantage compared

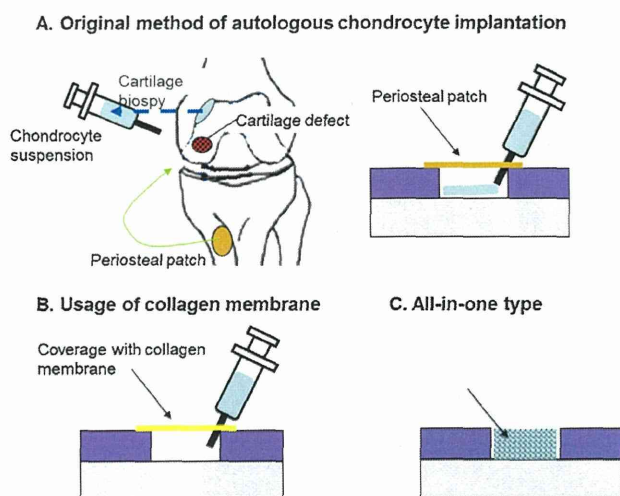

Fig. 5. Autologous chondrocyte implantation (ACI). Various types of ACI have been developed, including the original one (A), one with substitution of the periosteal patch with collagen membrane (B), and all-in-one type using animal-derived biomaterials (C).

with conventional treatment [11]. In Japan, the group of Professor Mitsuo Ochi of Hiroshima University developed a method of transplantation of chondrocytes embedded in collagen gel [12]. This technique was transferred to Japan Tissue Engineering Co., Ltd., and has been covered by health insurance from April 1, 2013 as autogenous cultured cartilage "JACC®." Regarding cartilage regenerative medicine in the maxillofacial region, subcutaneous cartilage regeneration has been used to treat patients in whom a silicone implant has been removed after augmentation rhinoplasty and those with saddle nose [13]. In these treatments, chondrocytes isolated from the patients' cartilage were expanded and injected into a subcutaneous pocket.

However, the present protocols for cartilage regenerative medicine either in the knee or in the nose utilize cells in solution or gel form, and thus are not sufficient for reconstruction requiring 3D morphological form.

6. Preclinical research on cartilage regenerative medicine

To develop tissue-engineered cartilage with 3D form and rigidity for the treatment of patients with severe nasal deformity associated with cleft lip and palate, we have conducted our research from the perspective of three elements of tissue engineering, that is, cells, growth factors, and scaffolds.

The choice of cells for cartilage regeneration varies from chondrocytes derived from cartilage tissue, to various cells classified as tissue stem cells, to embryonic stem cells (ES cells) and induced pluripotent stem cells (iPS cells). Of these, we considered that the use of autologous auricular chondrocytes would be practical and advantageous for early clinical application, because they: (1) proliferate vigorously in cell culture [14], (2) mature spontaneously after transplantation, (3) have a low risk of immune rejection or malignant transformation, (4) are relatively easy to harvest, and (5) pose no ethical problems.

We then examined factors that could efficiently induce the proliferation of human auricular chondrocytes. Of 12 growth factors/hormones confirmed to be safe as drugs (FGF-2, IGF-I, insulin, BMP-2, etc.), basic fibroblast growth factor (FGF-2; 100 ng/mL) showed the highest proliferation efficiency when added to 5% human serum as a single factor. Proliferation of chondrocytes could be further enhanced by combining insulin

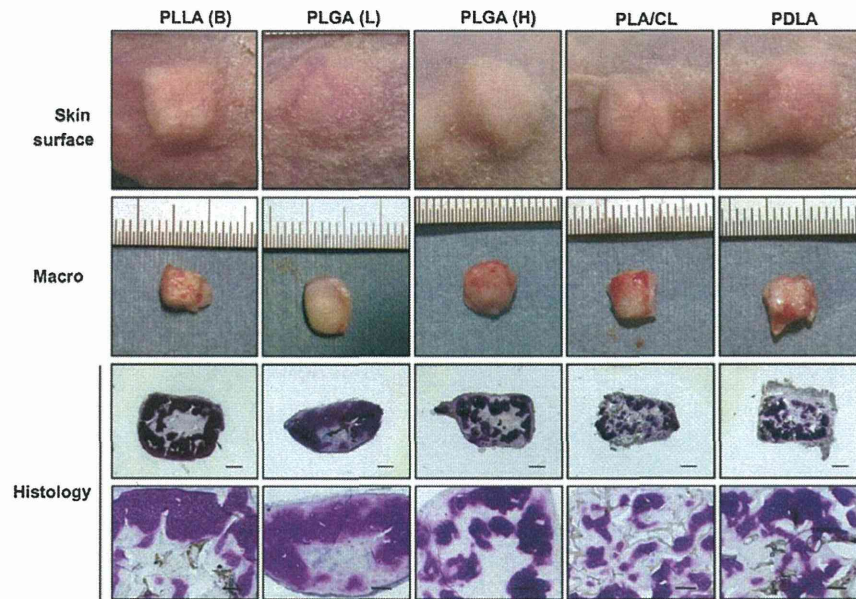

Fig. 6. Evaluation of scaffolds (porous material) for implant-type tissue-engineered cartilage (excerpted from Ref. [17] with permission). Macroscopic and histological images of tissue-engineered constructs made of porous scaffolds consisting of various biodegradable polymers with a chondrocytes/atelocollagen mixture with cell density of 1×10^8 cells/mL. The constructs (4 mm × 4 mm × 3 mm) were transplanted into the backs of nude mice. At 2 months in vivo, the skin surface was smooth with all the polymers. Based on the toluidine blue histological findings, metachromatic areas were more expansive than those in the experiment using 1×10^7 cells/mL. Macro and macroscopic images (bar = 2 mm); histology, bar = 1 mm (lower magnification) and 500 μm (higher magnification).

(5 μg/mL) with FGF-2 (100 ng/mL) and 5% human serum, realizing 1000-fold proliferation of human chondrocytes in 3 weeks [15].

Regarding scaffolds for tissue-engineered cartilage, we aimed to develop a composite scaffold consisting of hydrogel and a porous material that retains the advantages of both components. With respect to hydrogel, we have chosen atelocollagen gel, because it has a high cell-retaining capacity, promotes the expression and production of cartilage matrix, and is already being used clinically [16]. Meanwhile, to select a porous material, we compared several biodegradable polymers including poly-L-lactic acid (PLLA), poly-glycol acid (PGA), and poly-lactic acid/glycol acid (PLGA), which have been used clinically for plates, screws, and resorbable sutures. When evaluated in detail regarding pore size, porosity, and manufacturing method, PLLA and PLGA scaffold prepared by the sugar-leaching method with a pore size of 0.3 mm and porosity of 95% showed satisfactory mechanical strength. We then conducted animal experiments, in which human auricular chondrocytes in these scaffolds were transplanted subcutaneously in athymic nude mice. Histological and biochemical analysis demonstrated that the constructs using PLGA scaffold showed better cartilage regeneration (Fig. 6) [17]. However, in beagles, when auricular chondrocytes from 8-month-old male beagles were applied to PLGA scaffolds and transplanted subcutaneously into the back of the same animals, dense accumulation of macrophages was observed in tissue-engineered cartilage, and the tissue shrank after 2 months. In the case of using PLLA scaffolds, cartilage regeneration was found in the constructs 1 month after transplantation, and mature cartilage was observed after 6 months [18]. The reason why PLGA scaffold-based tissue-engineered cartilage, which showed satisfactory results in nude mice, failed in beagles may be related to the difference in tissue reaction against the two scaffolds. Compared to PLLA, degradation of PLGA is known to proceed rather rapidly after transplantation, releasing proinflammatory substances into tissues. Because beagles have a normal immune system, these substances may provoke excessive tissue reaction, inhibiting cartilage regeneration. On the basis of these evaluations, we have selected porous PLLA scaffold (pore size: 0.3 mm, porosity: 95%) for tissue-engineered cartilage that can be implanted for the treatment of patients with severe nasal deformity associated with cleft lip and palate (implant-type tissue-engineered cartilage).

7. Clinical application of implant-type tissue-engineered cartilage

Implant-type tissue-engineered cartilage consists of autologous chondrocytes that have been cultured in a medium containing 5% autologous serum and FGF2/insulin, atelocollagen gel (Koken, Tokyo, Japan), which has been approved as a medical device and as a PLLA porous scaffold (Fig. 7). PLLA porous scaffold has a domal structure with a length of 5 cm, width of 6 mm, and height of 3 mm, and has been used to treat nasal deformity associated with cleft lip and palate. Following a preclinical study confirming efficacy and safety, our protocol for implant-type tissue-engineered cartilage has been approved by the Institutional Review Board as well as the Ministry of Health, Labour and Welfare of Japan (MHLW). We have conducted clinical research on the patients with nasal deformity associated with cleft lip and palate at the Department of

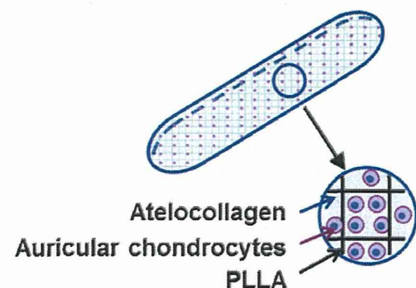

Fig. 7. Structure of implant-type tissue-engineered cartilage. It contains autologous chondrocytes, atelocollagen gel and a PLLA porous scaffold. The scaffold has a domal structure with length 5 cm, width 6 mm, and height 3 mm.

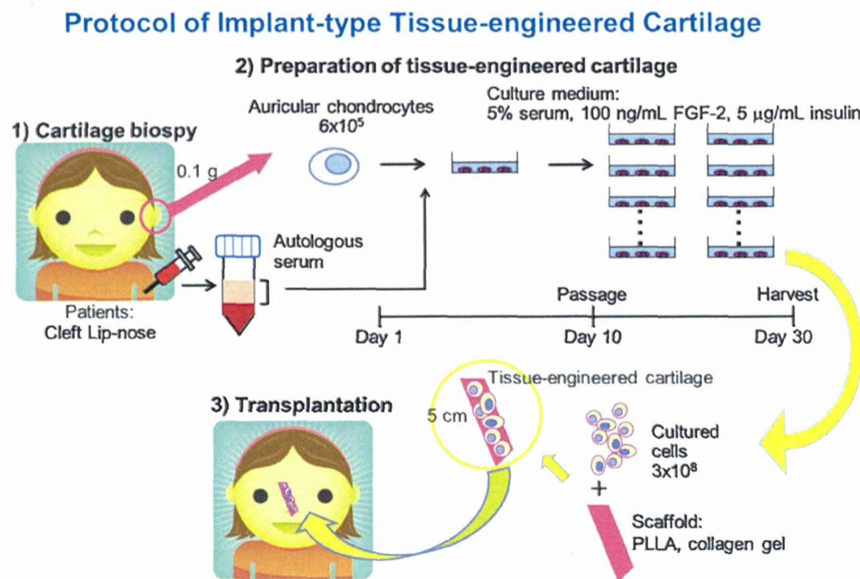

Fig. 8. Protocol of our clinical research on implant-type tissue-engineered cartilage. Auricular cartilage around 0.1 g is taken from patients, and then the isolated chondrocytes are cultured and expanded with a medium containing autoserum and FGF-2/insulin for a month. The cells are then administered into PLLA scaffold, and the construct is transplanted into the patient's nose.

Oral and Maxillofacial Surgery, University of Tokyo Hospital (Fig. 8). First, we obtained biopsy of auricular cartilage of around 0.1 g from the patients. The isolated chondrocytes were cultured with autoserum and FGF-2/insulin for a month. The cultured cells were then administered into the PLLA scaffold, and then the construct was transplanted into the patient's nose.

More than 2.5 years have passed since the first transplantation of tissue-engineered cartilage. Thus far, no serious adverse event requiring graft removal has been observed, and the clinical outcome has been satisfactory in all patients. We are now starting multicenter clinical research in cooperation with Tokyo Medical and Dental University and Yamagata University to assess evaluation methods for cartilage maturation, establish a transport system for tissue-engineered cartilage, and simplify the manufacturing process and quality control.

8. Development of implant-type regenerated cartilage

Implant-type tissue-engineered cartilage has mechanical properties as cartilage tissue and the desired morphological form can be achieved by adjustment of the shape of the scaffold. As it has been confirmed to be stable after transplantation in our clinical research, we are now planning to widen its indications to other tissues including the trachea and joints.

Regarding tissue-engineered trachea, we are considering its use for patients with tracheal stenosis. Autologous auricular chondrocytes are applied to a PLLA scaffold for cartilage regeneration, while micro-fabrication of the interior surface of a PLLA scaffold is expected to induce mucosal regeneration from surrounding tissues. We are evaluating the safety and efficacy of our tissue-engineered trachea in rabbits and beagles, and will perform an investigator-initiated clinical trial in six patients requiring tracheal reconstruction next year.

Regarding joints, we are developing a tissue-engineered joint with proper structure and sufficient strength that can be applied in patients with osteoarthritis. The construct is named NeoJoint; the cartilage part is prepared by administering cells in hydrogel into a PLLA scaffold, while the subchondral bone part is made of TCP artificial bone. At present, we are conducting autologous transplantation in Clawn miniature pigs using mesenchymal stem cells (MSCs) and iPS cells as the cell source. With either cell type, cartilage regeneration and bone repair have been observed on gross observation and histological analysis.

Our group has been engaged in the treatment of children with cleft palate and microtia for many years. In the reconstruction of microtia, the surgeon shapes ribs from the patient into an ear by the surgeons. Unfortunately, however, this operation is esthetically difficult, as well as invasive at the donor site, and thus we consider that the introduction of regenerative medicine would be beneficial. We hope that auricular reconstruction with a minimally invasive and esthetically satisfying technique using iPS cells and a scaffold prepared by 3D modeling will become possible.

Acknowledgments

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STEM CELLS

REGENERATIVE MEDICINE

Macrophage-Inducing FasL on Chondrocytes Forms Immune Privilege in Cartilage Tissue Engineering, Enhancing In Vivo Regeneration

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Key Words. Cartilage regenerative medicine • Chondrocyte • Macrophage • Fas ligand

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ABSTRACT

To obtain stable outcomes in regenerative medicine, controlling inflammatory reactions is a requirement. Previously, auricular chondrocytes in tissue-engineered cartilage have been shown to express factors related to immune privilege including Fas ligand (FasL) in mice. Since elucidation of mechanism on immune privilege formed in cartilage regeneration may contribute to suppression of excessive inflammation, in this study, we investigated the function of FasL and induction of immune privilege in tissue-engineered cartilage using a mouse subcutaneous model. When cocultured, auricular chondrocytes of FasL-dysfunctional mice, C57BL/6JSlc-gld/gld (gld), induced less cell death and apoptosis of macrophage-like cells, RAW264, compared with chondrocytes of C57BL/6 mice (wild), suggesting that FasL on chondrocytes could induce the apoptosis of macrophages. Meanwhile, the viability of chondrocytes was hardly affected by cocultured RAW264, although the expression of type II collagen was decreased, indicating that macrophages could hamper the maturation of chondrocytes. Tissue-engineered cartilage containing gld chondrocytes exhibited greater infiltration of macrophages, with less accumulation of proteoglycan than did wild constructs. Analysis of the coculture medium identified G-CSF as an inducer of FasL on chondrocytes, and G-CSF-treated tissue-engineered cartilage showed less infiltration of macrophages, with increased formation of cartilage after transplantation. The interactions between chondrocytes and macrophages may increase G-CSF secretion in macrophages and induce FasL on chondrocytes, which in turn induce the apoptosis of macrophages and suppress tissue reactions, promoting the maturation of tissue-engineered cartilage. These findings provide scientific insight into the mechanism of autologous chondrocyte transplantation, which could be applied as a novel strategy for cartilage tissue engineering. STEM CELLS *2014;32:1208–1219*

INTRODUCTION

Cartilage regenerative medicine using autologous chondrocyte transplantation (ACT) has been available since the 1980s, and more than 15,000 patients have received this treatment for focal cartilage defects in joints. Recently, some groups have expanded the indication for ACT to nasal augmentation, in which chondrocytes in a gelatinous chondroid matrix were injected into subcutaneous pockets in the nose [1]. We have also started a clinical study of ACT-based treatment using a biodegradable polymer for severe nasal deformity in patients with cleft lip and palate (JPRN-UMIN000005472). Since most of these cartilaginous diseases and deformities are irreversible and cannot be fully restored by conventional transplantation of autologous tissues or artificial materials, ACT-based treatment should be more prevalent. To broaden the application of ACT to severe defects in joint or maxillofacial cartilage, combined usage with a scaffold would be necessary to provide a proper morphologic shape and rigidity. Such scaffolds can be fabricated using biomaterials, such as biodegradable polymers.

ACT-based treatment uses autologous cells, and thus is advantageous to minimize the risk of immunogenic rejection. In studies using mice or beagles, however, when tissue-engineered constructs consisting of a biodegradable scaffold and syngenic or autologous chondrocytes were transplanted into subcutaneous pockets, some of them developed severe, prolonged tissue reactions [2, 3]. Histologically, accumulation of macrophages and abundant fibrosis were found in and around these transplants, with the formation of foreign body granulomas. Therefore, one of the requirements for successful transplantation of tissue-engineered cartilage is to control inflammation.

Meanwhile, in the case of successfully regenerated cartilage by transplanted

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chondrocytes in mice or beagles, chondrocytes were shown to produce local factors that could inhibit the actions of macrophages. In our previous study [2, 3], tissue-engineered cartilage constructs consisting of mouse auricular chondrocytes and a biodegradable scaffold were transplanted into enhanced green fluorescent protein (EGFP)-transgenic mice with the same genetic background. The host-derived cells, distinguished by the positivity of EGFP fluorescence, were mostly macrophages, and decreased and were confined to non-cartilage areas after an initial increase. The chondrocytes expressed some factors related to immune privilege including Fas ligand (FasL) and transforming growth factor-β, suggesting that chondrocytes in tissue-engineered cartilage may suppress the actions of macrophages by inducing immune privilege with these factors.

Immune privilege exists physiologically in certain tissues such as the eye, brain, ovary, testis, and pregnant uterus, where antigen normally does not initiate an immune reaction. The molecular mechanisms maintaining immune privilege in these tissues are considered to be a lack of lymphatic drainage, the presence of a physical barrier, and the production of immunosuppressive cytokines/neuropeptides [4]. Apart from the physiological immune privilege mentioned above, recent research has revealed another type of immune privilege, which is induced in pathological conditions. Hepatocytes do not express FasL, but FasL could be induced in hepatocellular carcinomas, promoting infiltration and growth in surrounding tissues [5]. Also, FasL becomes more detectable in hepatocytes in alcoholic hepatitis, liver allograft rejection, and Wilson's disease [6], suggesting that hepatocytes may have increased expression of FasL in pathological conditions. In another study, immunization was shown to induce the expression of functional FasL in the liver and small intestine [7], indicating that strong immune responses trigger the expression of FasL in some tissues. Such inducible peripheral, non-lymphoid FasL is considered to mitigate the tissue damage caused by strong immune response [4]. Considering that factors related to immune privilege were increased in tissue-engineered cartilage after transplantation in mice and beagles [2, 3], immune privilege may be induced in tissue-engineered cartilage as well by the surrounding immunological environment after transplantation.

In this study, we investigated the involvement of immune privilege in the transplantation of tissue-engineered cartilage, using mouse auricular chondrocytes. Among the mediators involved in immune privilege, we especially focused on FasL, and investigated its function in tissue-engineered cartilage using FasL-dysfunctional mice, C57BL/6JSlc-gld/gld (gld). The gld (generalized lymphoproliferative disease) mice are known to have a point mutation within the *Fasl* gene, resulting in the expression of nonfunctional FasL [8]. Furthermore, molecules that increased the expression of FasL on the auricular chondrocytes were identified, which were then applied in the subcutaneous transplantation of tissue-engineered cartilage in mice, to verify their effects in regulating tissue reactions and subsequent maturation of tissue-engineered cartilage.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F-12), penicillin-streptomycin solution, and trypsin-EDTA solution were purchased from Sigma Chemical Co. (St. Louis, MO, www.sigmaaldrich.com). Collagenase from *Clostridium histolyticum* was from Wako Pure Chemical Industries (Osaka, Japan, www.wako-chem.co.jp), insulin was from MP Biomedicals (Irvine, CA, www.mpbio.com), and FGF-2 was from Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan, www.kaken.co.jp). Other materials included 3% atelocollagen implants from Koken Co., Ltd. (Tokyo, Japan, www.kokenmpc.co.jp), poly(L-lactic acid) (PLLA) porous scaffolds from KRI (Kyoto, Japan, www.kri-inc.jp), anti-type II collagen antibody from LSL (Tokyo, Japan, www.cosmobio.co.jp), anti-F4/80 antibody (Cl: A3-1) from BMA (Augst, Switzerland, www.bma.ch), anti-fas ligand (FasL) antibody from GeneTex, Inc. (Hsinchu city, Taiwan, www.genetex.com), anti-STAT3 antibody, and anti-phospho STAT3 from Cell Signaling Technology Japan, K.K. (Tokyo, Japan, www.cellsignal.com). Recombinant mouse G-CSF, IL-6, IP-10, KC, MIP-1α, MIP-1β, and MIP-2 were from R&D Systems, Inc. (Minneapolis, MN, www.rndsystems.com). The biotinylated secondary antibody, Vectastain Elite ABC Kit, and Peroxidase Substrate kit 3,3′-Diaminobenzidine (DAB) were all obtained from Vector Laboratories (Burlingame, CA, www.vectorlabs.com). CytoTox 96 non-radioactive from Promega K.K. (Tokyo, Japan, www.promega.co.jp) and FITC Annexin V Apoptosis Detection Kit II from BD (Franklin Lakes, NJ, www.bd.com) were used. C57BL/6J (wild) and C57BL/6JSlc-gld/gld (gld) were purchased from Japan SLC, Inc. (Shizuoka, Japan, www.jslc.co.jp).

Cell Culture

Human cartilage was obtained under informed consent from remnant auricular cartilage of microtia patients who underwent surgery at Nagata Microtia and Reconstructive Plastic Surgery Clinic. All the procedures for these experiments were approved by the Ethics Committee (ethics permission #622, 752, and 753) or Institutional Committee for animal research of the University of Tokyo Hospital, and the experiments were conducted according to the principles expressed in the Declaration of Helsinki. Isolation of human auricular cartilage and culture of chondrocytes were conducted as previously described [9]. Mouse auricular chondrocytes were obtained as described previously [2]. Briefly, the ear and external ear canal of wild and gld mice were digested with 0.15% collagenase solution for 8 hours, and the isolated chondrocytes were cultured in DMEM/F-12 supplemented with 5% fetal bovine serum (FBS), 5 μg/ml insulin, and 100 ng/ml FGF-2. Both human and mouse auricular chondrocytes were cultured to passage 2. Mouse macrophage-like cell line RAW264 was purchased from the Riken Cell Bank (Tsukuba, Japan, www.brc.riken.jp) and maintained in MEM supplemented with 10% FBS and non-essential amino acids.

Fabrication of Tissue-Engineered Cartilage and Transplantation

PLLA scaffolds, which were produced by sugar-leaching method, were used. The molecular weight of the scaffolds was 200,000 average pore size was 0.3 mm, and average porosity was more than 95%. PLLA scaffolds of $4 \times 4 \times 3$ mm^3 were sterilized in 70% ethanol before use. Then, 1% atelocollagen gel, which was diluted from original 3% atelocollagen gel with DMEM/F-12, was used as a cell suspension buffer to retain the chondrocytes in the scaffolds efficiently. To make tissue-engineered constructs, mouse chondrocytes suspended in 1% atelocollagen gel (2×10^7 cells/200 μl) were applied to the PLLA scaffolds and incubated at 37°C in

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5% CO_2 for 2 hours. Regarding the transplantation procedure, wild mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). A small incision was made on the back in the midline, and two types of constructs, which contained either wild-derived or gld-derived chondrocytes, were transplanted subcutaneously in each animal. Two or eight weeks after the operation, the constructs were harvested and cut into equal parts ($n = 8$); one piece was frozen in liquid nitrogen and preserved at -80°C for biochemical analysis, and the other piece was fixed in 4% paraformaldehyde for 3 hours, embedded in paraffin, and cut into 8-μm sections for histological and immunohistochemical analysis. In some experiments, the constructs were treated with granulocyte-colony stimulating factor (G-CSF) (35 pg/ml) in redifferentiation medium [10] for 5 days, and then transplanted subcutaneously as described above.

Biochemical Analysis

Samples stored at -80°C for biochemical analysis were cut into small pieces with scissors, and suspended in 10 mg/ml pepsin and 0.05 M acetic acid. After digestion at 4°C for 48 hours, 1 mg/ml pancreatic elastase, 0.1 mM Tris, 0.02 M NaCl, and 5 mM $CaCl_2$ (pH 7.8–8.0) were added, and the samples were kept at 4°C overnight. The samples were then centrifuged at 9,100*g* for 5 minutes, and the sulfated glycosaminoglycan (GAG) content in the supernatant was measured using Alcian Blue binding assay (Wieslab AB, Lund, Sweden, www.wieslab.se) according to the manufacturer's instructions. Measurement was performed three times, and the mean values were used for statistical analysis.

Histological and Immunohistochemical Staining

The sections were stained with toluidine blue (TB) to detect proteoglycan as well as with hematoxylin and eosin (H&E staining). The sections were also used for immunohistochemical staining for F4/80, FasL, Fas, and CD3ε [9].

Cytotoxic Analysis and Apoptosis Detection

Mouse auricular chondrocytes (wild or gld) and a mouse macrophage cell line, RAW264, were cocultured at various ratios for 4 hours, and cytotoxicity was measured using CytoTox96 Non-Radioactive Cytotoxicity Assay. When activated macrophages were required, RAW264 was treated with lipopolysaccharides (LPS) (1 μg/ml) and IFN-γ (10 ng/ml) for 24 hours [11]. For the detection of apoptosis in macrophages, the cocultured cells (chondrocytes/RAW264 = 19:1) were stained with anti-F4/80 antibody, and the positivity of annexin V and propidium iodide (PI) in F4/80-positive cells was analyzed by flow cytometry using BD LSR II.

RNA Isolation and Real-Time RT-PCR

A mixture of mouse auricular chondrocytes and RAW264 (chondrocytes/RAW264 = 19:1) or chondrocytes alone in 1% atelocollagen gel were, respectively, embedded in PLLA scaffolds, and cultured in redifferentiation medium [10] for 7 days. Collected media were used for Proteome Profiler Array (R&D Systems, Inc., Minneapolis, MN, www.rndsystems.com), and secreted cytokines were quantified as the relative ratio by measuring the blotted areas. Subsequently, factors that increased in coculture by 100 relative ratios were chosen as possible inducers of FasL. Human auricular chondrocytes embedded in 1% atelocollagen gel (2×10^5 cells/20 μl) were cultured in redifferentiation medium containing the recombinant protein of the chosen factor. The concentration of each factor added to the medium was ED_{50}, which was provided by the manufacturers as well as 1/10th ED_{50} and 10 times ED_{50}. Five days later, total RNA was isolated with Isogen, and then reverse-transcribed with reverse transcriptase and random hexamers. Gene expression was detected by real-time RT-PCR using the standard SYBR green method with a 7500 fast real-time PCR system (Applied BioSystems, Carlsbad, CA, www.appliedbiosystems.com). Standard templates were produced according to a previous study [10]. Transcript levels were normalized to that of *GAPDH*.

Western Blotting

Human auricular chondrocytes embedded in 1% atelocollagen gel (2×10^5 cells/20 μl) were incubated for 2 hours before addition of 2 ml redifferentiation medium [10]. Some media contained G-CSF (35 pg/ml) and a JAK2 kinase inhibitor, AG490 (4×10^{-3} mol/l). After 10 and 30 minutes, the cells were harvested with M-PER, and the supernatant was used for Western blotting to detect the expression of STAT and p-STAT according to previous studies [10].

Statistics

Data are expressed as mean ± SD. Statistical significance was evaluated using Student's *t* test. A value of $p < .05$ was interpreted to denote statistical significance.

Results

Localization of Macrophages and Fas/FasL During In Vivo Cartilage Regeneration

We made tissue-engineered cartilage constructs using C57BL/6 mouse auricular chondrocytes, and syngenically transplanted them into the backs of host mice [2]. TB staining showed an increase in metachromatic areas by 8 weeks, indicating accumulation of proteoglycan and maturation of cartilage (Fig. 1A; TB). Immunohistochemical staining for COL2 also confirmed the maturation of transplanted cartilage (Fig. 1A; COL2). Meanwhile, F4/80-positive macrophages were prominent at 2 weeks, which then decreased and were localized in non-cartilage areas by 8 weeks (Fig. 1A; F4/80). FasL was immunolocalized on chondrocytes in the tissue-engineered transplants, and was especially intense at 2 weeks, the early stage of cartilage maturation (Fig. 1A; FasL). In contrast, cells positive for Fas were mainly macrophages, and were sparsely scattered throughout the observation period (Fig. 1A; Fas). We then made human tissue-engineered cartilage constructs and transplanted them into nude mice to examine the expression of *FASL* and *FAS* by real-time RT PCR using human-specific primers. *FASL* was enhanced in chondrocytes of tissue-engineered cartilage at 2 and 8 weeks after transplantation, while expression of *FAS* was decreased 8 weeks after transplantation (Fig. 1B; *FASL* and *FAS*). These findings suggest that FasL on chondrocytes may induce cell death in Fas-expressing macrophages and regulate tissue reactions.

Chondrocytes Reduce Viability of Macrophages

To examine the function of FasL expressed on chondrocytes, we prepared chondrocytes from C57BL/6J (wild) and FasL

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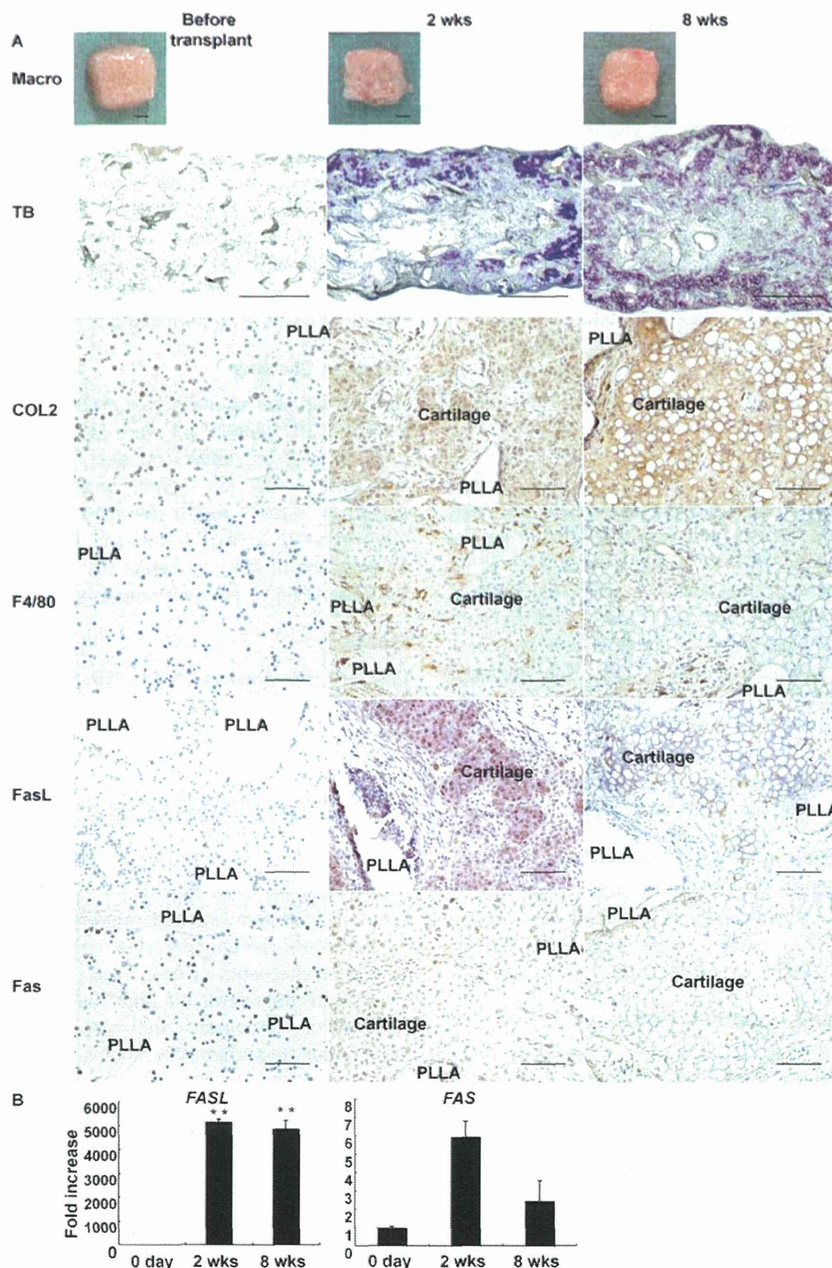

Figure 1. Expression of FasL and Fas in tissue-engineered cartilage constructs 2 and 8 weeks after transplantation. **(A):** (Macro) Macroscopic images of tissue-engineered cartilage. Scale bars = 1 mm. TB staining. Scale bars = 1 mm. (COL2, F4/80, FasL, and Fas) Immunohistochemical staining for COL2, F4/80, FasL, and Fas. Scale bars = 100 μm. **(B):** Expression of *FASL* and *FAS* in tissue-engineered cartilage was examined by real-time RT-PCR. Data are expressed as mean (bars) ± SD (error bars). **, $p < .01$, versus 0 day. Abbreviations: PLLA, poly(L-lactic acid); TB, toluidine blue.

hypomorphic mice, C57BL/6J-gld (gld). As macrophages have been identified as the major component of the host reaction [2], we cocultured chondrocytes with mouse macrophage-like cells, RAW264, at a ratio of 10 – 1.25:1, and examined the induction of cell death and apoptosis by cytotoxicity analysis and flow cytometry. Cytotoxicity analysis revealed that wild-chondrocytes induced more cell death in RAW264 than did gld-chondrocytes, when the ratio of chondrocytes to RAW264 was as high as 10:1 (Fig. 2A), suggesting that FasL on chondrocytes can induce cell death in cocultured macrophages. The difference in cytotoxicity of chondrocytes between wild and gld mice, however, became unrecognizable when the ratio of chondrocytes to RAW264 decreased. Since activated macrophages are known to have increased expression of Fas and thus be more apoptotic [12], we activated RAW264 with LPS and IFN-γ to see if this could increase the cell death of

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