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Use of Neonatal Chondrocytes for Cartilage Tissue Engineering

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Abstract Transplantation of cultured chondrocytes can regenerate cartilage tissues in cartilage defects in humans. However, this method requires a long culture period to expand chondrocytes to a large number of cells for transplantation. In addition, chondrocytes may dedifferentiate during long-term culture. These problems can potentially be overcome by the use of undifferentiated or partially developed cartilage precursor cells derived from neonatal cartilage, which, unlike chondrocytes from adult cartilage, have the capacity for rapid *in vitro* cell expansion and may retain their differentiated phenotype during long-term culture. The purpose of this study was to compare the cell growth rate and phenotypic modulation during *in vitro* culture between adult chondrocytes and neonatal chondrocytes, and to demonstrate the feasibility of regenerating cartilage tissues *in vivo* by transplantation of neonatal chondrocytes expanded *in vitro* and seeded onto polymer scaffolds. When cultured *in vitro*, chondrocytes isolated from neonatal (immediately postpartum, 2 h of age) rats exhibited much higher growth rate than chondrocytes isolated from adult rats. After 5 days of culture, more neonatal chondrocytes were in the differentiated state than adult chondrocytes. Cultured neonatal chondrocytes were seeded onto biodegradable polymer scaffolds and transplanted into athymic mice's subcutaneous sites. Four weeks after implantation, neonatal chondrocyte-seeded scaffolds formed white cartilaginous tissues. Histological analysis of the implants with hematoxylin and eosin showed mature and well-formed cartilage. Alcian blue/safranin-O staining and Masson's trichrome staining indicated the presence of highly sulfated glycosaminoglycans and collagen, respectively, both of which are the major extracellular matrices of cartilage. Immunohistochemical analysis showed that the collagen was mainly type II, the major collagen type in cartilage. These results showed that neonatal chondrocytes have potential to be a cell source for cartilage tissue engineering.

Key words: Neonatal chondrocyte, cartilage, tissue engineering

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Autologous chondrocyte implantation is an established technique to repair cartilage defect. In recent years, the procedure of autologous chondrocyte implantation to treat the defects in the articular surface of the knee joint has been widely applied in clinical use. In 1994, Brittberg and collaborators [3] introduced this procedure for the first time, which showed favorable clinical outcomes, such as the relief of symptoms and normal gross arthroscopic appearance of the treated lesion. Several additional studies have reported on the successful repair of cartilage defects by transplantation of cultured chondrocytes [4, 6, 11, 14, 15]. However, this method requires a long culture period to obtain a large number of chondrocytes expanded from a small cartilage biopsy.

Donor age and *in vitro* proliferative capacity of cultured chondrocytes may affect the properties of the cartilage regenerated by transplantation of the cultured chondrocytes. Several studies have shown that cartilage tissues regenerated by transplantation of chondrocytes from aged donors have smaller amounts of glycosaminoglycans and type II collagen than those regenerated by chondrocytes from young adult or fetus [16, 19]. This suggests that chondrocytes from aged donors affect negatively on the quality of the regenerated cartilage. Also, several additional studies showed that chondrocytes from aged donors have a limited growth potential *in vitro* [1, 2, 5, 8, 13, 17, 22]. Thus, it may require a long period to expand chondrocytes from aged donors to a large number of cells *in vitro*. When chondrocytes are maintained in monolayer culture for a long period, the cells eventually lose their differentiated phenotype [12, 21], and change their morphology from a polygonal or round shape to a flattened, amoeboid-like shape, and synthesize type I instead of type II collagen *in vitro* [20]. Therefore, chondrocytes from young donors may regenerate better quality of cartilage than those from aged donors.

Chondrocytes obtained from neonatal donors may have an advantage for cartilage tissue engineering. Neonatal chondrocytes may exhibit high proliferative capacity. Thus,

these cells may not require a long culture period to expand chondrocytes to a large number of cells for transplantation, avoiding chondrocyte dedifferentiation during the *in vitro* cell expansion. In this study, we investigated whether neonatal chondrocytes are more proliferative than adult chondrocytes, whether neonatal chondrocytes in culture maintain the differentiated phenotype better than adult chondrocytes, and whether transplantation of neonatal chondrocytes seeded onto polymer scaffolds regenerates cartilage tissues *in vivo*.

MATERIALS AND METHODS

Scaffolds

Biodegradable, nonwoven meshes fabricated from polyglycolic acid (Albani International Inc., Mansfield, MA, U.S.A.) fibers (12 μm in diameter) were utilized as three-dimensional scaffolds for cartilage tissue engineering. Before the use, the scaffolds were cut into squares (5×5 mm, 2 mm thickness), sterilized with ethanol, and washed with sterile distilled water.

Isolation and Culture of Chondrocyte

Chondrocytes were obtained from cartilages of the proximal tibia and distal femur from neonatal (immediately postpartum, 2 h of age, Fig. 1) and adult (9 to 10 months old) Sprague-Dawley rats. The cartilage fragments were minced, washed three times in phosphate buffered saline (PBS, Sigma, St. Louis, MO, U.S.A.), and digested in 0.25% (w/v) trypsin in PBS for 15 min, and then in 0.05% (w/v) collagenase type II (Sigma) in Dulbecco's Modified Eagle Medium/F-12 Ham (DMEM/F12, Gibco BRL, Grand Island, NY, U.S.A.) containing 10% (v/v) fetal bovine serum (FBS, Gibco BRL), 100 units/ml penicillin (Gibco BRL), and 0.1 mg/ml streptomycin (Gibco BRL) for 10 h. Recovered cells were washed in PBS and cultured in DMEM/F12 medium containing 10% (v/v) FBS, 100 units/ml penicillin, and 0.1 mg/ml streptomycin in a humidified 5% CO_2 incubator, with the medium changed every 3 days. The cell number was determined using a hemacytometer.

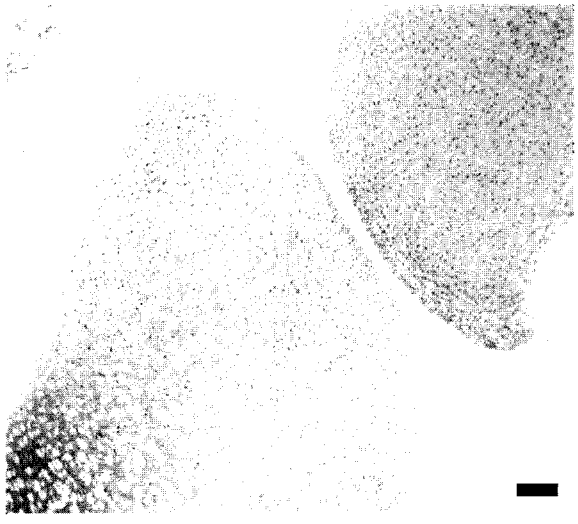

Fig. 1. Neonatal rat cartilages of the proximal tibia and distal femur as a cell source for chondrocyte isolation (safranin-O staining).
The size bar indicates 100 μm.

Cell Seeding onto Scaffolds and Implantation

Cultured neonatal chondrocytes (passage number=1) suspended (2×10^7 cells/ml) in DMEM/F12 were seeded onto PGA scaffolds (n=6). The chondrocyte-seeded scaffolds were maintained *in vitro* for 6 h at 37°C and implanted into subcutaneous dorsal spaces of 6 athymic mice (4-weeks-old male, SLC, Tokyo, Japan).

Analyses

For scanning electron microscopic examination of scaffolds seeded with neonatal chondrocytes and cultured for 6 h, the specimens were fixed in 1% (v/v) glutaraldehyde and 0.1% (v/v) formaldehyde for 30 min and 24 h, respectively, dehydrated with a series of graded ethanol, and dried. The dried samples were mounted on aluminum supports and sputter-coated with gold, and a scanning electron microscope (JEOL, Tokyo, Japan) was used to image the samples.

For histological analyses, specimens retrieved 4 weeks after implantation were fixed in 10% (v/v) buffered formalin, dehydrated with a series of graded alcohol, and embedded in paraffin. Tissue sections, 4 μm thick, were stained with hematoxylin and eosin (H&E) for morphologic analysis, alcian blue and safranin-O for sulfated glycosaminoglycans, and Masson's trichrome for cross-linked collagen. For immunohistochemical analyses, deparaffinized 4-μm thick sections were stained using antibodies raised against collagen

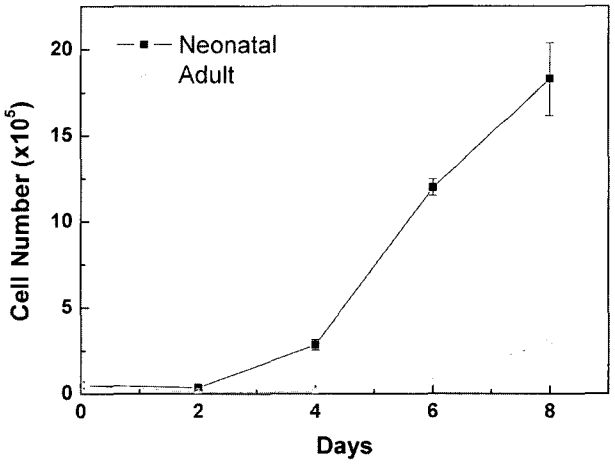

Fig. 2. Growth profiles of neonatal and adult chondrocytes in monolayer culture.

types I and II (Chemicon, Temecula, CA, U.S.A.). The staining signal was developed with an avidin-peroxidase system (ABC kit, Vector Laboratory, Burlingame, U.S.A.). Gills hematoxylin (Sigma) was used for counterstaining.

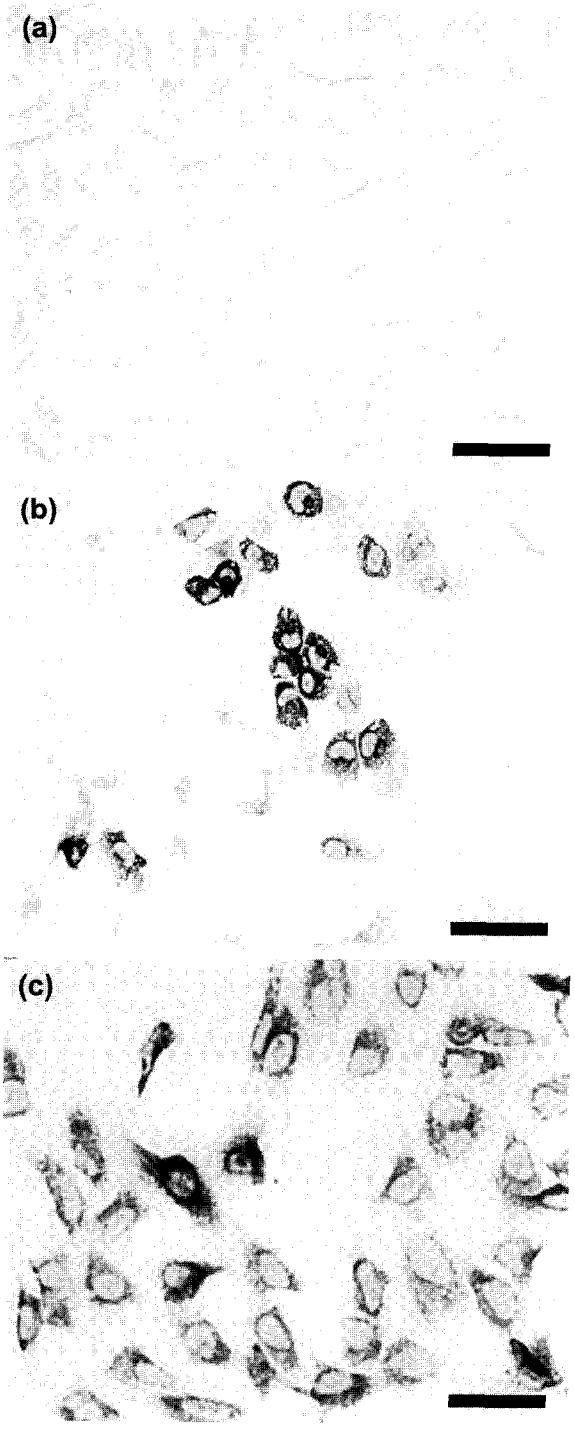

Fig. 3. Photomicrographs of cultured rat chondrocytes on day 5. (a) Neonatal chondrocytes with polygonal morphology. Immunostaining for collagen type II of (b) neonatal chondrocytes and (c) adult chondrocytes in culture. The size bars indicate 50 μm.

RESULTS AND DISCUSSION

Neonatal chondrocytes exhibited much higher growth rate than adult chondrocytes when cultured *in vitro* (Fig. 2). After 8 days of *in vitro* cell expansion, the number of neonatal chondrocytes was approximately 6-fold higher than that of adult chondrocytes. The cultured neonatal cells

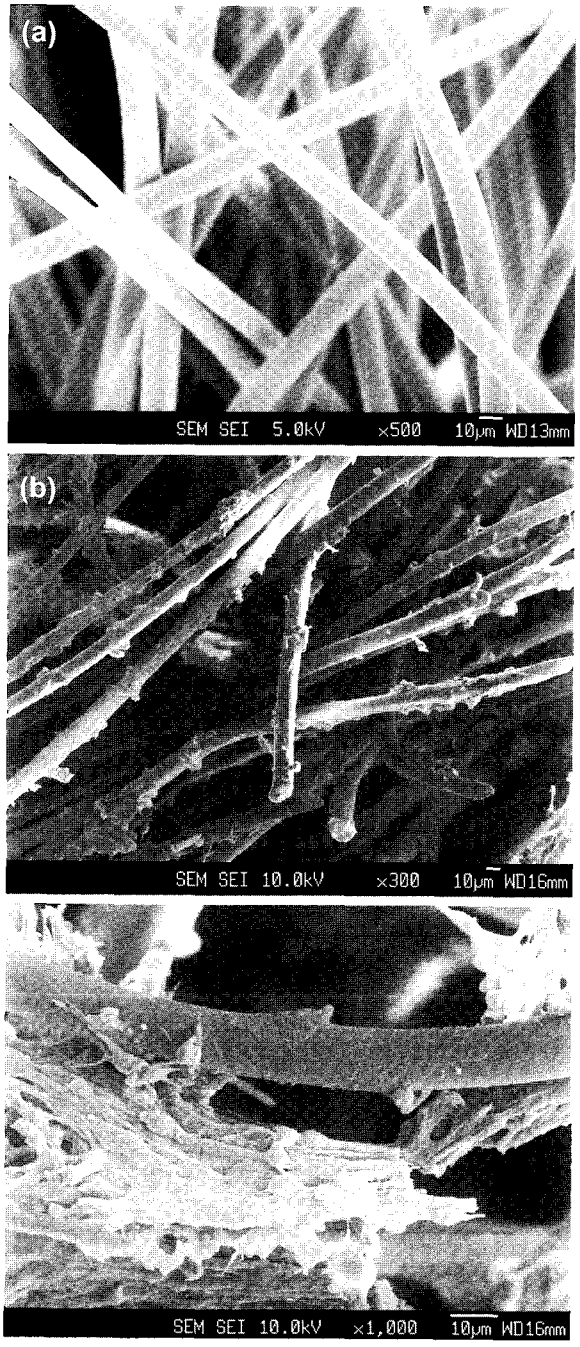

Fig. 4. Scanning electron microscopic photographs of (a) PGA scaffold and (b, c) neonatal chondrocyte-seeded scaffold (b: ×300; c: ×1,000).

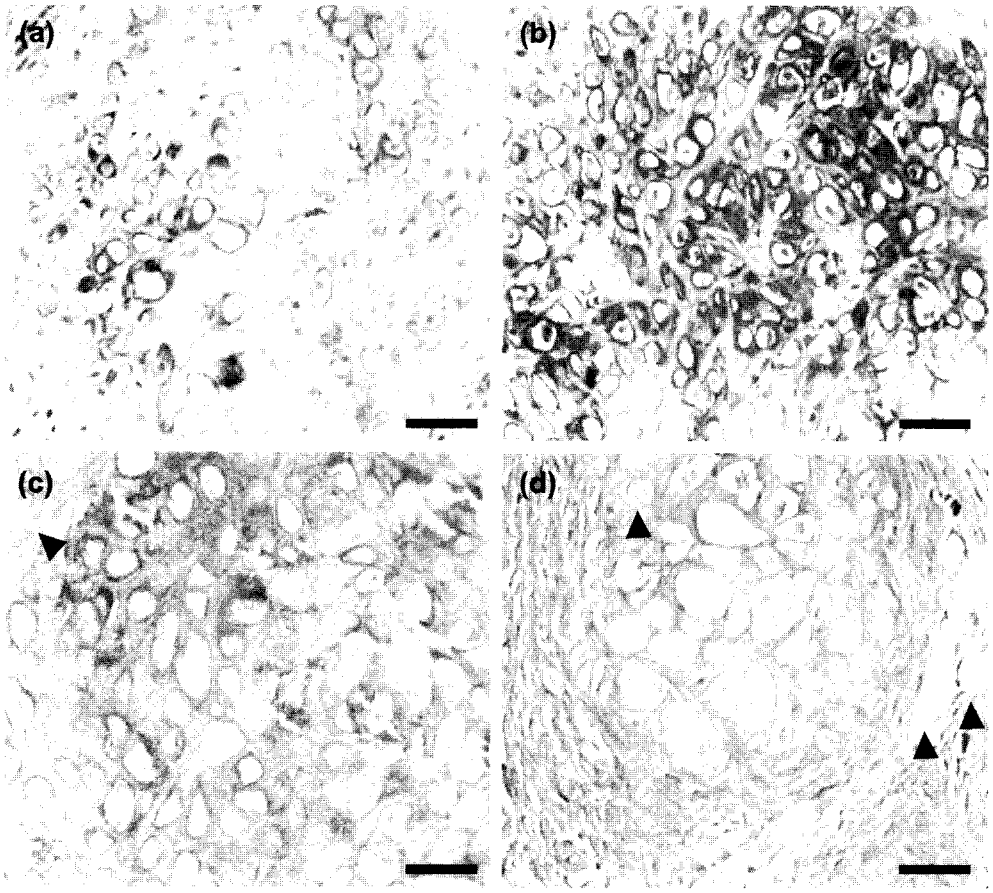

Fig. 5. Histological sections of neocartilages formed by implantation of neonatal chondrocyte-PGA scaffold constructs for 4 weeks. (a) H&E staining, (b) safranin-O staining, (c) alcian blue staining, and (d) Masson's trichrome staining. The arrowheads indicate PGA fiber. The size bars indicate 50 μm.

showed a polygonal shape, which is a characteristic of chondrocytes (Fig. 3a). On day 5 of culture, most neonatal chondrocytes stained positively for collagen type II, the major collagen type produced by differentiated chondrocytes, whereas many adult chondrocytes stained negatively for collagen type II (Figs. 3b, 3c). This suggests that neonatal chondrocytes have a higher tendency to maintain the differentiated phenotype, when cultured *in vitro*, than adult chondrocytes.

The seeding of neonatal chondrocytes on three-dimensional polymer scaffolds resulted in the formation of three-dimensional chondrocyte-polymer constructs. PGA meshes served as a porous scaffold (Fig. 4a), which provides a substrate for cell adhesion and three-dimensional space for cartilage tissue formation upon implantation. Six hours after seeding, scanning electron microscopic examination of the chondrocyte-seeded scaffolds revealed that the chondrocytes adhered well on the polymer scaffolds (Figs. 4b, 4c).

Four weeks after transplantation, neonatal chondrocytes-seeded PGA scaffolds formed milk-white cartilaginous tissues *in vivo*. When retrieved, chondrocyte-PGA constructs were easily visualized under the dorsal skin of the athymic mice. The new tissues appeared to be avascular. The original shape of the chondrocyte-PGA constructs was approximately maintained.

Histological analysis with H&E staining of the implants retrieved at 4 weeks showed that implanted chondrocytes regenerated mature and well-formed cartilage, as evidenced by chondrocytes in lacunae (Fig. 5a). The neocartilage was avascular. There was no sign of inflammation or fibrotic layer formation around the newly-formed cartilage tissues. Safranin-O (Fig. 5b) and alcian blue (Fig. 5c) stainings indicated the presence of highly sulfated glycosaminoglycans, which are extracellular matrices produced by differentiated chondrocytes. Masson's trichrome staining showed the presence of collagen in the regenerated cartilage (Fig. 5d). Immunostaining for type II collagen was positive, prominently in the matrices adjacent to lacunae (Fig. 6a). Staining for type I collagen was negative (Fig. 6b). This suggests that the neocartilage contained the right type of collagen.

Chondrocytes obtained from neonatal donors may be advantageous over adult chondrocytes as a cell source for

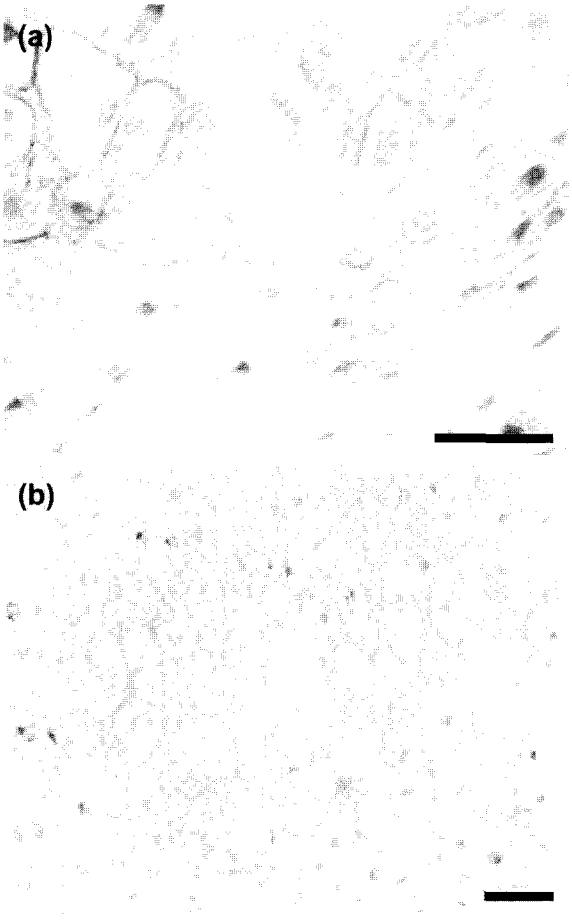

Fig. 6. Immunohistochemical stainings for collagen (a) type II and (b) type I of neocartilages formed by implantation of neonatal chondrocyte-PGA scaffold constructs for 4 weeks. The size bars indicate 50 μm.

cartilage tissue engineering. Neonatal chondrocytes in culture may proliferate faster, require shorter culture period to expand to a large number of cells, and show higher tendency to maintain differentiated phenotype than adult chondrocytes. Chondrocyte density is many times higher in neonatal cartilage than in adult cartilage [18]. Thus, a large number of chondrocytes can be obtained more easily from a small neonatal cartilage biopsy than from adult cartilage biopsy. In addition, several reports have shown that neonatal cells have lower immunologic reactions when transplanted, compared with their adult counterparts [7, 9, 10].

In summary, this study demonstrated the feasibility of regenerating cartilage tissues *in vivo* by transplanting neonatal chondrocytes. Neonatal chondrocytes exhibited more rapid cell expansion *in vitro* than adult chondrocytes. During the cell culture, neonatal chondrocytes had higher tendency to maintain the differentiated phenotype than adult chondrocytes. Implantation of neonatal chondrocytes seeded onto polymer scaffolds resulted in the formation of three-dimensional cartilage tissues. The neocartilage displayed the histological characteristics of hyaline cartilage. Biochemical quantification, such as of glycosaminoglycans and type II collagen, and the mechanical properties of neocartilage must be characterized in order to evaluate the quality of neocartilage. Additional studies, including transplantation to immune-competent animals and long-term studies on transplantation, would help toward a better appreciation of the clinical potential of this approach.

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