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Gamma-cross-linked nonfibrillar collagen gel as a scaffold for osteogenic differentiation of mesenchymal stem cells

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We fabricated a transparent nonfibrillar collagen gel using gamma irradiation (5 kGy) and cultured rat mesenchymal stem cells (MSCs) on both the gamma-irradiated collagen gel and on unirradiated fibrillar collagen gel. Cells attached well and proliferated with high viability on the surface of both gels. The cells cultured on the gamma-irradiated nonfibrillar gel had a unique elongated shape and adhered to each other in culture. After 21 days of culture in dexamethasone-containing culture medium, the contents of bone-specific osteocalcin and calcium on the gamma-irradiated nonfibrillar gel were 1.4 and 1.9 times higher than those on fibrillar collagen gel, respectively. These data show that osteogenic differentiation of MSCs was promoted more efficiently on the gamma-cross-linked nonfibrillar gel than on the fibrillar gel and demonstrate the potential of the gamma-irradiated collagen gel for use in bone tissue engineering.

[**Key words:** Collagen fibril; Bone formation; Osteoblast; Bone marrow stromal cell; Mesenchymal stem cell]

Bone is composed of both collagen fibrils and hydroxyapatite, and then forms the skeleton keeping the shape of vertebrate body. Bone is formed and resorbed by osteoblasts and osteoclasts, respectively (1). Osteoblasts, which are differentiated from mesenchymal stem cells (MSCs), deposit hydroxyapatite on the surface of Type I collagen fibrils to form new bone, and then differentiate into osteocytes (2–5). Osteoclasts, which are characteristic multinuclear cells differentiated from hematopoietic stem cells (HSCs), resorb bone.

Studies on bone regeneration attract much attention in the orthopedic research field and also in the tissue engineering research field (1–7). It is the most important to efficiently induce the differentiation of MSCs into osteoblasts those deposit hydroxyapatite on the collagen fibrils in bone tissue engineering. Type I collagen is abundant in connective tissues including dermis, bone, tendon and ligament, and is also the protein most widely used as a scaffold in tissue engineering (8). Various scaffolds consisting of type I collagen have been used in tissue engineering (5) and play very important roles in controlling differentiation of MSCs into different cell types by interacting with the cells to transduce signals via integrins (9–11) and also by transducing mechanical signals (12). Tissue engineering of hard tissues such as bone and cartilage requires use of a scaffold suitable for efficient induction of differentiated cells from the MSCs. Usually chemical or physical crosslinking techniques are used to reinforce the collagen gel or collagen sponge because the collagen gel itself is rather fragile (8,13).

We previously studied a gamma-ray crosslinked type I collagen hydrogel (13) and reported the method for preparation of a novel collagen gel without formation of collagen fibrils by strong gamma-ray irradiation (14,15). Our study focused on the biological activity of this novel collagen gel without collagen fibrils. We recently reported that HOS cells showed good osteogenic activity and deposited calcium phosphate on a gamma-cross-linked nonfibrillar gel (16). HOS cells are an experimental model strain of osteoblasts (17–19), however the cells were derived from an osteosarcoma and not from genuine osteoblasts. Viable osteoblasts can be generated by culturing MSCs, especially in culture medium containing dexamethasone, and the differentiated osteoblasts are capable of in vitro bone formation (2,3). We therefore performed comparative studies of in vitro bone formation on both the gamma-cross-linked nonfibrillar gel and the unirradiated fibrillar gel containing collagen fibrils by culturing rat mesenchymal stem cells (MSCs) and found that the former gel promotes the osteoblastic differentiation of MSCs. We propose that gamma-cross-linked nonfibrillar gel is a promising scaffold for osteoblastic differentiation of MSCs in bone tissue engineering in this paper.

MATERIALS AND METHODS

Materials Porcine type I collagen (Collagen BM, type I, 0.6% (w/v), pH 3.0, Nitta Gelatin, Osaka, Japan), and dexamethasone (Dex, 111-07, Nacalai Tesque, Kyoto,

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Japan) were used. Other reagents used in our experiments were the same as described previously (16). A 25 × stock solution of PBS(−) was prepared by dissolving 5 g KCl, 5 g KH_2PO_4, 72.4 g $Na_2HPO_4 \cdot 12H_2O$, and 200 g NaCl in 1 L of distilled water and then autoclaving to sterilize. The solution was diluted to prepare PBS(−) or 10 × PBS(−).

Preparation of collagen gels The fibrillar collagen gel and the gamma-cross-linked nonfibrillar gel [described as neutral gel and acidic gel, respectively, in our previous paper (16)] used for the cell culture experiments were both prepared aseptically as described below. For the fibrillar collagen gel, aliquots of acidic collagen solution (700 μL/well) and 10 × PBS(−) (78 μL/well) were added to each well of a 24-well plate and incubated for 2 h at 37°C to form a white fibrillar collagen gel, which was used for cell culture without irradiation. For the gamma-cross-linked nonfibrillar gel an acidic collagen solution (4.0 mL/well) in a 6-well plate was irradiated by γ-irradiation at a dose of 5 kGy (dose rate; 8.97−9.38 kGy/h) at room temperature, in the Radiation Research Center of Osaka Prefecture University using the ^{60}Co (350 TBq) γ-ray source. The gamma-cross-linked nonfibrillar gels formed in the wells were then incubated in culture medium for 48 h at 37°C while they shrank, then the shrunk gels were transferred into 24-well plates. The size of the shrunk gels could be adjusted to fit wells of a 24-well plate by the procedure described previously (16).

Isolation of MSCs and cell culture conditions The medium used to culture rat MSCs was MEM containing 15% (v/v) fetal bovine serum (FBS) and 1% (v/v) Antibiotic-Antimycotic (AB). Isolation of the MSCs was performed as described previously (2,20). A male inbred Fisher 344 rat (7 weeks) was subjected to a lethal overdose of the anesthetic, diethylether, in a sealed glass chamber. A femur was removed from the hind limb, and both ends of the femur were cut off using a scalpel under aseptic conditions on a clean bench. The bone marrow was washed out of the femur into a disposable plastic test tube by flushing with culture medium from the needle of a syringe inserted into the femur. The marrow from two femurs was then collected in a 50 mL test tube and cultured for 1 week in a T-75 flask containing 15 mL medium at 37°C under 5% (v/v) CO_2. Cells adhered on the bottom of the flask were cultured, passaged twice, and then used for the experiments as MSCs. Non-adherent cells in the bone marrow were mostly removed by these procedures.

Staining of actin filaments for image analysis of cell morphology Cultured MSCs at day 1 were fixed in a 4% (w/v) paraformaldehyde (PFA) solution for 30 min at 4°C, washed with PBS to remove residual PFA, and then permeabilized in 0.3% (v/v) Triton X-100. Specific staining of intracellular actin filaments was achieved by incubation for 30 min with 100 nM rhodamine-labeled phalloidin (Cytoskeleton, Denver, CO, USA) according to the standard protocol. The stained cells were observed using a fluorescent microscope (Optiphoto-2, Nikon, Tokyo, Japan) and images were recorded using a digital camera (DS-5Mc, Nikon). Images were manipulated in a personal computer (PC) to calculate five parameters about the cell shapes as described below using LabVIEW software (National Instruments, Austin, TX, USA). Fig. 3D illustrates the concept of the cell parameters including the area of stained actin filaments (μm^2), cell area (μm^2), perimeter of the cell (μm) and ratio of (maximal Feret diameter):(minimal Feret diameter). The Feret diameter is a measure of an object's size along a specified direction, a length of the edge of a circumscribed rectangle, and is therefore also called the caliper diameter. The linear elongation shape factor (E) was defined as follows:

$$E = F/F_R = F^2/A \quad (1)$$

where F is the maximal Feret diameter, F_R is the shortest side of the rectangle with the same area as the cell and A is the area of the cell.

Roundness of the cells was defined as follows:

$$R = C/P = 2^*\mathrm{SQRT}(\pi A)/P \quad (2)$$

where C is the circumference of an equivalent circle with the same area as the cell and P is the perimeter of the cell. Images with a resolution of 0.14 μm/pixel were used for the calculation to obtain these parameters.

Osteogenic culture of MSCs MSCs (1.0×10^5 cells/mL) in 1 mL of medium were inoculated into each well of a 24-well plate, with either the fibrillar gel or the gamma-cross-linked nonfibrillar gel, prepared as described above. Two types of medium were used. Dex(−) medium consisted of MEM containing 15% (v/v) FBS, 1% (v/v) AB, 10 mM β-glycerophosphate (β-GP) and 82 μg/mL Vit. C. The Dex(+) medium was the same as Dex(−) with the addition of 10 nM dexamethasone (Dex), which triggers osteogenic differentiation of the MSCs (2,3).

Evaluation of the cultured MSCs Live/dead staining, alkaline phosphatase (ALP) staining, Alizarin Red S staining, and calcein staining of MSCs on both the

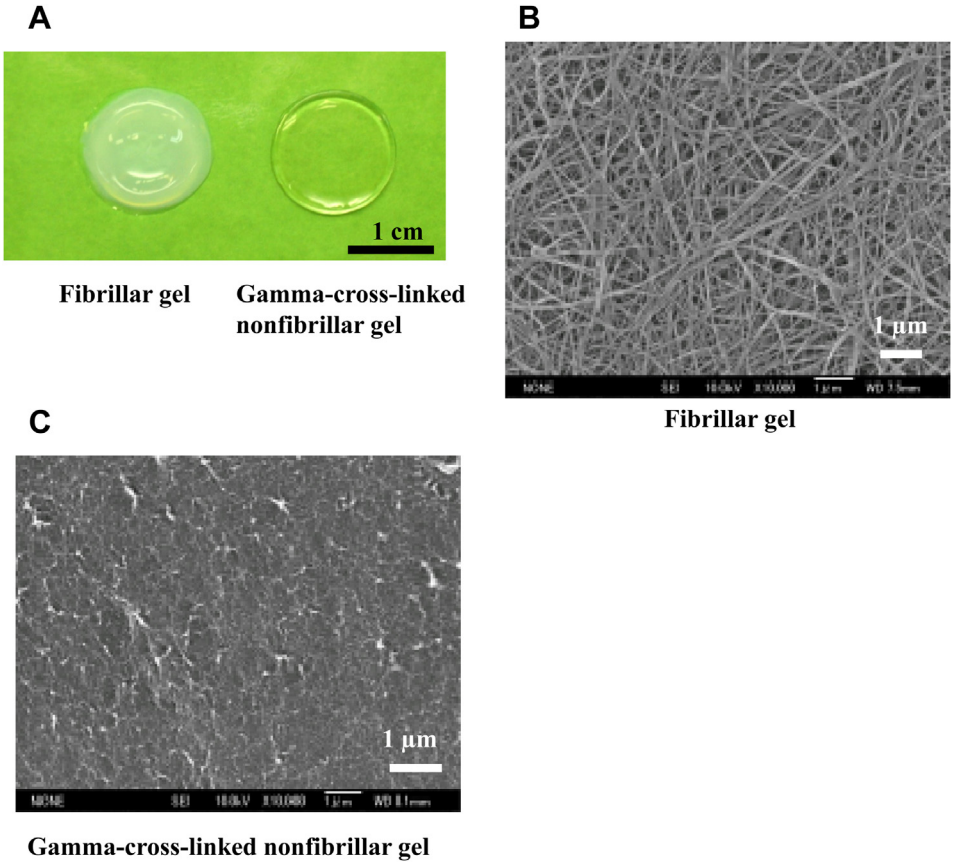

FIG. 1. Photomicrographs of the fibrillar collagen gel (A, left), and the gamma-cross-linked nonfibrillar gel (A, right). SEM images of the fibrillar gel (B) and the gamma-cross-linked nonfibrillar gel (C). Scale bar represents 1 cm in panel A and 1 μm in panels B and C.