Journal of Bioscience and Bioengineering
VOL. 122 No. 2, 219–225, 2016

www.elsevier.com/locate/jbiosc

Chondrogenic differentiation of human mesenchymal stem cells on fish scale collagen

Han-Hsiu Hsu,[1] Toshimasa Uemura,[1,§,*] Isamu Yamaguchi,[2] Toshiyuki Ikoma,[3] and Junzo Tanaka[3]

Department of Life Science and Biotechnology, Biotechnology Research Institute for Drug Discovery, National Institute of Advanced Industrial Science and Technology, 1-1-1 Higashi, Tsukuba Central-5, Ibaraki 305-8562, Japan,[1] Taki Chemical Co. Ltd., 346 Miyanishi, Harima-cho, Kako-gun, Hyogo 675-0145, Japan,[2] and Department of Metallurgy and Ceramics Science, Tokyo Institute of Technology, S7-6, 2-12-1 Ookayama, Meguro-ku, Tokyo 152-8550, Japan[3]

Received 7 September 2015; accepted 4 January 2016
Available online 30 January 2016

Fish collagen has recently been reported to be a novel biomaterial for cell and tissue culture as an alternative to conventional mammalian collagens such as bovine and porcine collagens. Fish collagen could overcome the risk of zoonosis, such as from bovine spongiform encephalopathy. Among fish collagens, tilapia collagen, the denaturing temperature of which is near 37°C, is appropriate for cell and tissue culture. In this study, we investigated chondrogenic differentiation of human mesenchymal stem cells (hMSCs) cultured on tilapia scale collagen fibrils compared with porcine collagen and non-coated dishes. The collagen fibrils were observed using a scanning electronic microscope. Safranin O staining, glycosaminoglycans (GAG) expression, and real-time PCR were examined to evaluate chondrogenesis of hMSCs on each type of collagen fibril. The results showed that hMSCs cultured on tilapia scale collagen showed stronger Safranin O staining and higher GAG expression at day 6. Results of real-time PCR indicated that hMSCs cultured on tilapia collagen showed earlier SOX9 expression on day 4 and higher AGGRECAN and COLLAGEN II expression on day 6 compared with on porcine collagen and non-coated dishes. Furthermore, low mRNA levels of bone gamma-carboxyglutamate, a specific marker of osteogenesis, showed that tilapia collagen fibrils specifically enhanced chondrogenic differentiation of hMSCs in chondrogenic medium, as well as porcine collagen. Accordingly, tilapia scale collagen may provide an appropriate collagen source for hMSC chondrogenesis *in vitro*.

[**Key words:** Fish scale collagen; Chondrogenesis; Mesenchymal stem cells; Extra-cellular matrix; Tilapia collagen]

Type I collagen, a kind of extracellular matrix, plays an important role in cell culture and enhances cell adhesion, cell extensibility, and cell growth (1). Many kinds of mammalian collagens are now available commercially, however, there is a risk that diseases such as bovine spongiform encephalopathy (BSE) and foot-and-mouth disease (FMD) could be transmitted via materials from mammalian sources. Fish collagen has been suggested as an appropriate alternative to mammal collagens such as porcine- and bovine-derived collagen due to the low risk of zoonotic infection from fishes to humans (2). In addition, fish collagen is commercially available as foods and cosmetic materials with a ready supply of low-cost fishery waste providing a stable source of fish collagens (3).

Fish scales consist of hydroxyapatite and type I collagen in an almost 1:1 ratio (4,5). Collagen can be prepared at high purity from fish scales compared with from fish skin or other fishery waste (3). However, depending on the habitat temperature of each species of fish, the denaturing temperatures of fish collagens, such as sea bream (29.9°C) (5), salmon (19°C) (6), chub mackerel (25.5°C) (7), and bullhead shark (25°C) (7), are generally lower than mammals collagens (37–42°C). Here we focus on tilapia (*Oreochromis niloticus*, a tropical fresh-water teleost) scale collagen whose denaturing temperature is about 37°C (5).

Tilapia is farm-raised for food in East Asia and whose scales are easily collected as low-cost fishery waste. Fish scales have a high regeneration potential (8,9), and tilapia scale collagen is highly oriented and has the potential for rapid fibril formation (9). Osteoblastic and osteogenic differentiation of human mesenchymal stem cells (hMSCs) has been reported to be accelerated on tilapia scale collagen by our group (3,10). hMSCs were also reported to have multi-potency for differentiation into bone, cartilage, muscle, fat (11,12), nerve (13), and liver (14) tissues. From this point of view, we focused on chondrogenesis of hMSCs on tilapia collagen. We compared the morphology of tilapia and porcine collagen when coated on dishes, and examined cell growth and chondrogenic differentiation of hMSCs on tilapia collagen, porcine collagen and non-coated dishes. A chondrogenic marker, glycosaminoglycans(GAGs) (15), was used to evaluate chondrogenesis of hMSCs. The comparative CT method of quantitative real-time PCR was used to evaluate mRNA expression levels of SOX9, AGGRECAN, COLLAGEN II, and bone gamma-carboxyglutamate protein (BGLAP) in hMSCs. Here we show that tilapia scale collagen could be used as an appropriate scaffold for hMSC chondrogenesis *in vitro*.

MATERIALS AND METHODS

Collagen observation by scanning electron microscopy Tilapia collagen was prepared using Cell Campus AQ-03A (Taki Chemical, Hyogo, Japan; 0.3%, pH 3.0). Porcine collagen were prepared with Cellmatrix I-C (Nitta Gelatin, Osaka, Japan;

* Corresponding author. Tel./fax: +81 6 6879 4973.
E-mail address: uemura@prec.eng.osaka-u.ac.jp (T. Uemura).
§ Present address: Department of Precision Science and Technology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan.

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2016.01.001

0.3%, pH 3.0) for comparison. For scanning electron microscopy (SEM) observation, tilapia and porcine collagen solution were adjusted to 0.2 ml 0.1% collagen/PBS and added to a 1.5 ml tube and incubated at 30°C for 3 h. Gel-like collagen fibrils were treated with 4% paraformaldehyde (Sigma–Aldrich, SL, USA) for fixation, and exchanged into 30%, 50%, 70%, 90%, 95%, and 100% ethanol (Wako Pure Chemical Industries, Osaka, Japan), sequentially, for dehydration. Samples were isolated, air-dried, mounted by gold coating using a sputter coater (Van Loenen, Hilversum, Netherland), and observed by SEM (Hitachi Ltd., Tokyo, Japan).

Preparation of collagen-coated dishes For dish coating, 0.3% collagen solutions from tilapia and porcine were added into 6-well non-tissue-culture-treated dishes (Falcon, MA, USA; 351143) and stood for 2 h below 25°C. Collagen solutions were removed from the dishes, which were air dried for 30 min, followed by three washes with PBS (Wako Pure Chemical Industries). The dishes were air dried below 25°C before use. Due to the denaturation temperature of both collagens (Table 1), all operations are done under 25°C.

Cell culture hMSCs (lot. no. 6F3974, Lonza Walkersville, MD, USA) were grown in mesenchymal stem cell growth medium (MSCGM, Lonza Walkersville) to passage 4, trypsinized with 0.25% trypsin-EDTA (Lonza Walkersville), washed with Dulbecco's modified Eagle's medium (D-MEM; high-glucose, Wako Pure Chemical Industries) containing 10% fetal bovine serum (FBS), and centrifuged at 1000 rpm for 1 min before use. Precipitated cells were maintained in MSCGM and seeded on tilapia collagen, porcine collagen, or non-coated wells in 6-well plates (Falcon) at 10^4 cells/cm^2 per well. After culture for 20 h, the medium was changed to hMSC chondrocyte differentiation medium with hMSC Chondrogenic Single Quots (Lonza Walkersville) containing 10 ng/ml TGF-β3 (Wako Pure Chemical Industries) (day 0). Subsequently, the medium was changed every 3 days.

Safranin O staining Safranin O staining kit (IHC World, MD, USA) was used for staining of chondrogenic matrices, with slight modifications. Cells cultured on collagen-coated or non-coated dishes for 8 days with hMSC Chondrogenic Single Quots (Lonza Walkersville) medium containing 10 ng/ml TGF-β3 were washed with PBS and fixed with 10% formalin (Wako Pure Chemical Industries) for 10 min. Cells were dehydrated subsequently with 70% and 95% ethanol for 1 min, followed by dehydration in 100% ethanol for 10 min. The ethanol was discarded and cells were stained using Weigert's Iron Hematoxylin solution for 10 min with shaking. Cells were washed by PBS and acetic acid solution for 15 s, which was then discarded very quickly. Cells were stained with Safranin O solution for 10 min with shaking, washed with PBS, and observed using EVOS Cell Imaging System (Life Technologies, CA, USA).

Cell suspension After culturing, cells were isolated using 0.0002% papain solution which consisted of papain (Sigma Aldrich) and suspension buffer containing 0.1 M sodium acetate, 0.01 M Na_2EDTA, 0.005 M cysteine HCl, 0.2 M Na_2HPO_4, and 0.2 M NaH_2PO_4. Briefly, cells in each well are detached with 1 ml 0.0002% papain solution at 37°C for 1 min. The suspension was collected in 1.5 ml tubes, put on ice for 5 min, and stood at 65°C for 3 h. Tubes were centrifuged at 10,000 *g* for 10 min, and the cells were collected for the GAG and DNA assays.

Glycosaminoglycan and DNA assays A Blyscan Sulfated Glycosaminoglycan Assay kit (Biocolor, YF, UK) was used to evaluate GAG expression secreted from hMSCs. Briefly, 1 ml dye reagent was added to 0.5 ml of cell suspension in 1 ml tubes and shaken for 30 min, followed by centrifugation at 10,000 ×*g* for 10 min. The supernatant was removed and 0.2 ml dissociation reagent was added to disrupted the pellets. Then 0.05 ml of each mixture was added to a 96-well plate (Falcon) and analyzed using a Versa Max microplate reader (Molecular Devices, CA, USA) at a wavelength of 656 nm (n = 3). Using a GAG standard curve with concentrations of 20, 15, 10, 5 and 0 μg/ml, the concentration of GAG in each sample was calculated. A PicoGreen dsDNA Assay kit (Invitrogen, MA, USA) was used to determine the total DNA amount in cultured hMSCs in accordance with the manufacturer's protocol. Then, the GAG/DNA ratios were calculated.

Total RNA extraction and real time PCR Total RNA from the hMSCs was extracted using an RNeasy Micro kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. cDNA was synthesized from 600 ng of total RNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems, MA, USA) using an iCycler (Bio-Rad, CA, USA), and adjusted to 400 ng/μl. PCR reactions contained cDNA templates and components of TaqMan Fast Universal PCR Master Mix (Applied Biosystems). Primers for SOX9 (Hs00165814_m1), AGGRECAN (Hs00153936_m1), COLLAGEN II (Hs00264051_m1), and bone gamma-carboxyglutamate protein (BGLAP) (Hs01587814_g1) (TaqMan Gene Expression Assays, Applied Biosystems) were used. Real-time PCR amplification was performed using the comparative CT (ΔΔCT) method, with pre-initiation at 50°C for 2 min, initial denaturation at 95°C for 20 s, and 40 cycles of annealing and extension at 60°C for 20 s. Data were normalized with the data for GAPDH (Hs99999905_m1) expression in cells from non-coated dish, by calculating the ratio of target gene/GAPDH expression in each sample. All reactions were run in triplicate.

Data analysis and statistics All experiments were repeated three times, and representative data were analyzed using Student's paired *t* test for comparison between each two groups. Values are shown as the mean ±SD. *P* values <0.05 were considered significant, and *P* values <0.001 were considered statistically significant.

TABLE 1. Amino acid composition (residues (%)) and gelation temperatures of tilapia scale and porcine dermis collagen.

Residues (%)	Tilapia scale	Porcine dermis
Hydroxyproline	8.3	9.7
Aspartic acid	4.7	4.4
Threonine	2.4	1.6
Serine	3.6	3.3
Glutamic acid	7.2	7.2
Proline	11	12.3
Glycine	34.6	34.1
Alanine	12.6	11.5
Half-cystine	0	0
Valine	2	2.2
Methionine	1.2	0.6
Isoleucine	1.1	1
Leucine	2.1	2.2
Tyrosine	0.3	0.1
Phenylalanine	1.3	1.2
Hydroxylysine	0.7	0.7
Lysine	2.5	2.7
Histidine	0.5	0.5
Arginine	4.9	4.8
Gelation temperatures	18.2°C	23.5–37°C

RESULTS

Morphology of tilapia scale and porcine collagen fibrils To compare collagen fibrils formed from tilapia scale and porcine collagen molecules, both collagen fibrils were observed by SEM. Tilapia collagen formed fibrils of 1.0–2.5 μm diameter (Fig. 1A), whereas porcine collagen formed fibrils of 0.5–1.5 μm diameter (Fig. 1B). Images also showed that tilapia collagen fibrils looked helically coiled (Fig. 1A), whereas porcine collagen showed a simpler structure with individual fibrils (Fig. 1B).

Safranin O staining of hMSCs on tilapia and porcine collagens hMSCs were pre-cultured on tilapia and porcine collagen-coated dishes and non-coated dishes (control) for 20 h, then cultured with chondrogenic differentiation medium for 8 days. To observe chondrogenesis, the results of Safranin O staining of hMSCs on day 0 and day 8 are shown in Fig. 2. At day 0, no chondrogenic differentiation was observed for any of three treatments (Fig. 2A–C), whereas on day 8, the images of hMSCs on tilapia and porcine collagen were positive in Safranin O staining (Fig. 2D, E, G, H). Collagen-coated dishes showed better cell attachments and proliferation, compared with non-coated dishes (Fig. 2F, I). More-obvious red color was observed on tilapia collagen (Fig. 2D, G) compared with porcine collagen (Fig. 2E, H), suggesting that tilapia collagen may provide a better extracellular matrix for chondrogenesis.

GAG/DNA of hMSCs on tilapia and porcine collagens Based on the above qualitative results, to quantitatively analyze chondrogenesis of hMSCs on tilapia and porcine collagens, GAG and DNA were isolated from cells and evaluated as shown in Fig. 3. Total GAG was isolated from hMSCs cultured on tilapia or porcine collagen on days 0, 2, 4, 6, and 8 (Fig. 3A). Samples from non-coated dishes were used as controls. Compare to porcine collagen and the control, hMSCs on tilapia collagen showed significant higher total GAG expression on day 6 (Fig. 3A). To evaluate GAG expression by cell proliferation, the total DNA amount in cultured cells on days 0, 2, 4, 6, and 8 were analyzed (Fig. 3B). GAG/DNA quantification of hMSCs cultured on tilapia collagen, porcine collagen, or non-coated dishes after treatment to induce chondrogenesis for 0, 2, 4, 6, and 8 days were calculated (Fig. 3C). According to the results, temporal changes of hMSC GAG/DNA on tilapia and porcine collagen can be observed from day 2 to day 8. Firstly, tilapia samples showed significantly lower GAG/DNA on day 2 compared with porcine samples (Fig. 3C). Afterward, significantly