INTRODUCTION

Three-dimensional culture is required to regenerate tissues and organs of interest through cell differentiation. Many three-dimensional culture techniques utilize a cell mixture in a scaffold. Porcine collagen has traditionally been used as a scaffold and put to practical use in skin and mucosal models. We have conducted various studies on ES cell differentiation using porcine atelocollagen. However, even if tissues and organs can be generated in three-dimensional culture in collagen derived from mammals, such as pigs, there remain problems, such as BSE and viruses. Fish are considered to be free of these problems, because humans and pigs are mammals, while fish are species distinct from mammals. Extraction Technology of Collagen from tilapia scales has been developed. We previously cultured mouse ES cells using collagen derived from

SYNOPSIS

Porcine atelocollagen has traditionally been used as a scaffold for cell culture in the field of regenerative medicine. Marine collagen, rather than collagen derived from mammals has recently attracted attention. However, the collagen of fish (e.g., salmon) that inhabits cold oceans has already been demonstrated to be unsuitable as a three-dimensional scaffold for long-term culture at 37°C. In the present study, we examined whether type I collagen, extracted from the scales of tilapia inhabiting tropical and subtropical areas, can be used as a scaffold for ES cells. As a result, it had no effect on ES cell differentiation as compared with conventional porcine collagen. In a previous study, salmon collagen could not withstand long-term culture. Thus, the collagen obtained from tilapia, featuring a high denaturation temperature (35-37°C), is useful as a scaffold for ES cell differentiation.

Key words: ES cells, 3D culture, Tilapia, scaffold, collagen
imai et al., Mouse ES Cell Differentiation using Collagen Derived from Tilapia Scale

salmon, which inhabits cold oceans, as marine collagen. Differentiating ES cells into tissues and organs of interest requires continuous long-term culture. However, a scaffold using salmon collagen posed no problem in about 1-week culture at 37°C, but precluded continuous culture for three weeks or one month because of collagen denaturation 13. It has already been demonstrated that cell growth should be increased for cultured cells, and this tilapia collagen facilitates early osteoblastic differentiation of human mesenchymal stem cells 17. We examined whether this collagen can be used as a scaffold for the long-term culture of mouse ES cells.

MATERIALS AND METHODS

1. Cells
The embryonic stem cells from the D3 mouse cell line (ES-D3 cells) and clone A31 from mice (Fig. 1).

2. Assay medium
Preheat treated 20% (v/v) fetal calf serum (FCS, HyClone®, Lot. AUH35508, Utah, USA) was added to 1% (v/v) nonessential amino acids (NAA, Invitrogen, CA, USA), 0.1 mM β-mercaptoethanol (Invitrogen), 2 mM L-glutamine (Invitrogen), and Dulbecco's Modified Eagle Medium (DMEM, Nacalai Tesque, Kyoto, Japan) with penicillin / streptomycin (Invitrogen). Except during tests, 1,000 U/mL of mouse leukemic inhibitory factor (mLIF, 10⁻⁷ unit, ESGRO®, Millipore, CA, USA) was added to inhibit for free differentiation.

3. Differentiation assay
ES-D3 cells were diluted in assay medium to a final concentration of 3.75 × 10⁴ cells/mL using a hemacytometer, and a 20 μL cell suspension was dropped 40-60 times onto the inside of the lid of a 10 cm diameter Petri-dish using a micropipette. Each drop of the cell suspension contained approximately 750 cells. Five mL of sterilized phosphate buffered saline (PBS, Invitrogen) was poured into the Petri-dish, and the lid was quickly reversed and placed on the dish before the cell suspension on the lid could flow down. Suspension culture was carried out for three days in a CO₂ incubator (5% CO₂ and 95% air; 37°C). Drops of the cell suspension on the inside of the Petri dish lid were then collected into a 6 cm diameter dish for germiculture. The mediums were replaced with new ones using a pipette and each mediums was subjected to reaction for two days in the CO₂ incubator.

4. Three-dimensional culture
Marine collagen (TC, AQ-03A, TAKI Chemical Co., Ltd. Hyogo, Japan, Fig.2) and porcine collagen were used as scaffolds. Porcine type I-A collagen alone (CG, Cellmatrix, Nitta Gelatin Inc. Osaka, Japan) and a mixture of type I-A and III collagens (3:1) were used. Under ice cooling, 8 mL of each collagen, 1 mL of medium supplemented with 10-fold concentration Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, MO, USA), Non-Essential Amino Acids (Invitrogen), and a 20 μL cell suspension was dropped 40-60 times onto the inside of the lid of a 10 cm diameter Petri-dish using a micropipette. Each drop of the cell suspension contained approximately 750 cells. Five mL of sterilized phosphate buffered saline (PBS, Invitrogen) was poured into the Petri-dish, and the lid was quickly reversed and placed on the dish before the cell suspension on the lid could flow down. Suspension culture was carried out for three days in a CO₂ incubator (5% CO₂ and 95% air; 37°C). Drops of the cell suspension on the inside of the Petri dish lid were then collected into a 6 cm diameter dish for germiculture. The mediums were replaced with new ones using a pipette and each mediums was subjected to reaction for two days in the CO₂ incubator.
trogen), β-mercaptoethanol (Invitrogen), and 0.5% (v/v) L-glutamine (Invitrogen) and 1 mL of reconstruction buffer.

Three types of collagens of 300 μL were put in the cell culture inserts (Pore size=1.0 μm, BD Biosciences, FALCON, NJ, USA), and into the assay medium were pipette into the outside of the cell culture inserts. The embryoid bodies (EBs) was placed on collagen gel matrix (Fig. 3). The cell culture inserts were transferred into the dishes, in which fresh assay medium was added, followed by 5-day culture in the incubator. Pulsating cells that differentiated into cardiomyocytes were observed with an inverted phase microscope (MD-70, Olympus, Tokyo). Differentiation rate were determined from the percentages of wells containing pulsating cells among all wells.

RESULTS

After 1-week culture, TC, CG, and MG were 67, 64, and 70%, respectively. No significant difference was noted in the mean values. Even after 2-week culture, no significant difference was noted in the beating rates of the heart muscle. After 3-week culture, no significant difference was noted between TC and MG (63% for TC, 50% for CG, and 62% for MG), although the beating rate of the heart muscles was reduced for CG (Fig. 4). Beating heart muscle due to EB differentiation in teratoma was microscopically observed. Inverted phase-contrast microscope images of teratoma...
Marine and porcine collagens posed no problem in the 2-week EB culture. However, after 3-week culture, CG was slightly reduced. Type I-A collagen alone produced a slightly hard gel. Thus, the entire EB could not sink into the gel, resulting in poor three-dimensional culture conditions. Deterioration of the medium outside intercell can be disregarded because the medium was replaced every three days for all collagens.

Collagen is a protein with a triple-helical structure in vivo. Recently, marine collagen has become commercially available as supplements and cosmetics. Various kinds of fish are included (e.g., salmon, flounder, cod, and sea bream). Fish that inhabit tropical or subtropical areas have collagen with a high denaturation temperature because they inhabit water.

**DISCUSSION**

Marine and porcine collagens posed no problem in the 2-week EB culture. However, after 3-week culture, CG was slightly reduced. Type I-A collagen alone produced a slightly hard gel. Thus, the entire EB could not sink into the gel, resulting in poor three-dimensional culture conditions. Deterioration of the medium outside intercell can be disregarded because the medium was replaced every three days for all collagens.

Fig. 4 ES cell differentiation rate of three types collagen.
Determine the number of wells with contracting areas for each well.

Fig. 5 Phase-contrast microscope images of teratoma after 3-week culture. Morphological images of teratoma in three types collagen gel was not observed in the microscope image.

After 3-week culture are shown in Figure 5.
at around 37°C. Thus, the collagen can withstand long-term culture like mammalian collagen. Generating tissues and organs from ES or iPS cells in vitro is likely to require considerably long-term culture. Differentiating mouse ES cells into any organ requires a 3-week or longer culture period. Human cell differentiation requires a longer culture period. Thus, generating tissues and organs in vitro may require a novel technique to shorten the cell differentiation period. However, accelerating cell differentiation may cause different problems, such as malignant transformation. The collagen extracted from the scales of tilapia is likely to serve as a promising scaffold for tissue regeneration.

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