Title:

Activities of MC3T3-E1 Cells Cultured on γ-Irradiated Salmon Atelocollagen Scaffold

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ABSTRACT

We investigated the effect of γ-irradiation on the activities of MC3T3-E1 cells cultured on γ-irradiated salmon atelocollagen (SAC) scaffolds. SAC-cultured samples were irradiated at doses of 10, 15, and 25 kGy. γ-Irradiation had a significant impact on the activities of MC3T3-E1 cells. The proliferation rates and alkaline phosphatase activities of MC3T3-E1 cells increased with γ-irradiation dose.

Collagen is one of the major components of the animal extracellular matrix and has important functions providing mechanical strength in tissues and a scaffold for cell growth and differentiation (1). In humans, 21 types of collagen have been described (2). Collagen molecules are made of three polypeptides known as α-chains and have a unique triple-helix configuration of three α-chains (3). Type I collagen represents the most abundant protein in the body and the majority of collagen materials for biomedical applications. Type I collagen (abbreviated as collagen) has non helical telopeptides at both ends of its molecules, which serve as the major source of antigenecity (4). Atelocollagen, which is produced by the elimination of telopeptides using pepsin, demonstrates low antigenecity, nontoxicity, biocompatibility, and biodegradability. Therefore, atelocollagen is used for the repair and regeneration of tissues, such as epidermis (5), neurons (6), muscle (7), cartilage (8), liver (9), bone tissues (10), by tissue engineering.

Collagen is primarily obtained from bovine skin. The use of domestic animals in medicine, however, is being reconsidered because of the risk for the transmission of infectious diseases to human beings such as bovine spongiform encephalopathy (BSE). We have therefore been studying the application of fish skin collagen in biomaterials (11-13). Fish skin is thought to have a lower risk than bovine skin for transmitting

diseases and to be a potentially large source of collagen (14). In spite of such advantages, fish skin collagen has not been extensively studied, except shark skin collagen, due to its low denaturation temperature (15). The denaturation temperature of fish skin collagen is lower than the body temperature of humans. Thus, fish skin collagen melts when placed in contact with the human body. We consider that cross-linking among collagen molecules increases the low stability of fish skin collagen.

Recently, we have reported stabilization methods for salmon atelocollagen (SAC) and the application of SAC to the scaffold of human periodontal ligament (HPDL) cells (12). SAC was extracted from salmon skin discarded as waste in the food industry. Thus, the use of SAC could contribute to the recycling of unutilized resources. SAC is sensitive to heat and melts at 19ºC owing to its low hydroxyproline content compared with that of bovine collagen (16). SAC can be stabilized by cross-linking it with a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (11, 13). EDC was found to be a low-toxicity reagent because EDC is not incorporated in peptide bonds and the by-product of EDC cross-linking is urea, which has zero cytotoxicity and can be easily removed by rinsing (17). HPDL cells proliferate and differentiate well on an EDC-cross-linked SAC scaffold (SAC-EDC scaffold) (12). In this study, we investigate the use of the SAC-EDC scaffold for implant medical

devices for the regeneration therapy of periodontal and bone tissues.

Sterilization is essential for implant medical devices. The routinely used sterilization processes are high-pressure steam (autoclaving) and dry heat treatments. These processes, however, cannot be used for heat- or water-sensitive biomaterials such as collagen. Ethylene oxide sterilization is extensively used to sterilize heat-sensitive medical devices. However, the toxic gas residue that remains in such devices becomes a serious hazard to patients (18). γ-Irradiation is the simplest and most effective method of sterilization without the generation of toxic substances. Few studies have shown the effects of γ-irradiation on cell activities, and the physical and biological properties of the collagen scaffold. γ-Irradiation leads to the degradation of poly(L-lactide) (PLLA) and the degraded PLLA molecules stimulate the calcification of MC3T3-E1 cells (19). In addition, a significant number of peptide bonds are cleaved by the irradiation which can cause considerable changes in the long-term characteristics of collagen scaffolds (20). The effect of γ-irradiation on the activities of the cells cultured on that irradiated collagen scaffold therefore has to be investigated before using such a method in the sterilization of implant devices.

In this study, MC3T3-E1 cells were cultured on SAC-EDC scaffolds γ-irradiated at various doses (0, 10, 15, and 25 kGy), and the proliferation rate and alkaline

phosphatase (ALP) activity of the cells were examined. In addition, the degree of cross-linking of the SAC-EDC scaffold was measured.

SAC-EDC scaffolds were prepared as reported previously (12). A lyophilized SAC was added to 100 ml of diluted HCl (pH 3) to a concentration of 0.5% (w/v) and stirred overnight at 4ºC to give SAC solution. Next, 1 ml of SAC solution was poured into multiple 24-well plates for tissue culture (Asahi Techno Glass, Tokyo), which was kept at -70ºC for 24 h. The plates were then placed in a lyophilizer (FDU-830; EYELA Tokyo Rikakikai, Tokyo) for 24 h. The lyophilized scaffolds were immersed in 4 M NaCl aqueous solution including 1% (w/v) EDC (reagent grade; Dojindo Laboratories, Kumamoto) and placed at 4ºC for 24 h. The cross-linked scaffolds were washed three times with diluted water and then lyophilized again.

The SAC-EDC scaffolds were irradiated at room temperature using ^{60}Co γ-rays, in the Japan Radioisotope Association Koka laboratory (Shiga). The irradiation doses were 0, 10, 15, and 25 kGy.

MC3T3-E1 cells were obtained from the Riken Cell Bank (Ibaraki). MC3T3-E1 cells were cultured in α-minimal essential medium (α-MEM; Sigma Aldrich Japan, Tokyo) containing 10% fetal bovine serum (FBS; Gibco BRL, Life Technologies, Rockville, MD, USA) at 37ºC in air containing 5% CO_2. The culture medium was

changed every 2 d. At semiconfluence, MC3T3-E1 cells were subcultured in the same medium. The cells passaged less than 18 times were used for cell culture on the irradiated samples. The irradiated samples were preincubated in α-MEM containing 10% FBS for 1 h. Then, MC3T3-E1 cells were seeded on the SAC-EDC scaffolds at a density of 5×10^4 cells/cm^3 and cultured in multiple 24-well plates (Asahi Techno Glass, Tokyo). The medium was changed every 2 d. After specific time periods, the proliferation rate and ALP activity of the MC3T3-E1 cells were measured as described below.

Cell proliferation rate was analyzed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay (CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay; Promega, Tokyo) (12). The cultured scaffolds were rinsed with phosphate-buffered saline (PBS) three times, and 1 ml of 5 mg/ml MTS was added. After incubation for 2 h, absorbance was measured at 492 nm using a microplate reader (Spectrafluor Plus; TECAN Japan, Tokyo).

To clarify the function of differentiated MC3T3-E1 cells, ALP activity was determined. The scaffolds were washed three times with PBS and 400 μl of lysis buffer (0.5% Triton X-100, 150 mM NaCl, 10 mM HEPES) was added. The lysate was

incubated for 30 min at 4ºC and centrifuged (2000×*g*). Then 100 µl of 0.02 M $MgCl_2$ in 0.1 M Tris-HCl (pH 8.8) and 4 mg/ml *p*-nitrophenylphosphate (Sigma Aldrich Japan KK, Tokyo) solution in 0.2 M Tris were mixed and preincubated at 37ºC. Then 100 µl of the cell lysate was added to the mixture and incubated at 37ºC for 30 min. The absorbance of liberated *p*-nitrophenol was measured at 405 nm with a spectrophotometer. ALP activity was normalized with MTS absorbance.

The degree of cross-linking was determined using 2,4,6-trinitro-benzensulfonic acid (TNBS, analytical grade; Wako Pure Chemical Industries, Osaka) as previously described (12). To a sample of 3-5 mg of the SAC-EDC scaffold, 1 ml of 4% (w/v) $NaHCO_3$ solution (pH 8.5) and 1 ml of freshly prepared 0.5% (w/v) TNBS solution in distilled water was added. After incubating for 2 h at 40ºC, 2 ml of 6 N HCl was added, and the temperature was raised to 60ºC and the sample was solubilized for 1 h. The absorbance of the resulting solution was measured at 345 nm with a spectrophotometer (U-2001; Hitachi, Tokyo). The degree of cross-linking was calculated using a previously reported formula (12). Experiments were performed in triplicate. All data are expressed as mean ± the standard deviation (SD) for n = 3.

The proliferation rates of MC3T3-E1 cells are shown in Fig. 1. There was a remarkable difference between the proliferation rates after 7 d cultivation. The

proliferation rates of MC3T3-E1 cells were highest at the doses of 15 and 25 kGy. At the dose of 10 kGy, the proliferation rate was intermediate between that of nonirradiated samples and that of 15 kGy- or 25 kGy-irradiated samples. γ-Irradiation significantly increased the proliferation rates of MC3T3-E1 and proliferation rate increased with irradiation dose.

The ALP activities of MC3T3-E1 cells are shown in Fig. 2. γ-Irradiation significantly increased the ALP activities of MC3T3-E1 cells. There was no remarkable difference in ALP activity among the three γ-irradiation doses of 10, 15, and 25 kGy.

The degrees of cross-linking of the SAC-EDC scaffolds are shown in Fig. 3. The degree of cross-linking of the nonirradiated sample was 59.1% and those of the samples irradiated at the doses of 10, 15, and 25 kGy were 66.6%, 65.1%, and 72.5%, respectively. These results indicate that γ-irradiation increases the degree of cross-linking of collagen molecules.

The activities of the cells cultured on the irradiated samples were affected by γ-irradiation. The proliferation rates and ALP activities of MC3T3-E1 cells cultured on the irradiated samples increased with γ-irradiation dose. There were no changes in the morphology of the irradiated samples before and after γ-irradiation (data not shown), whereas there were changes in the degree of cross-linking (Fig. 3). Nishikawa *et al.*

have reported that the cross-linking of collagen enhances the cell proliferation of epidermal cells and fibroblasts (21). The increase in the degree of cross-linking appears to be one of the reasons for the increase in cell activities. However, the mechanism of this increase in cell activities caused by cross-linking is still unclear. There might be some changes in the physical properties of the γ-irradiated samples caused by cross-linking in addition to the change in the degree of cross-linking. No enzymatic degradation of the γ-irradiated samples with collagenase or trypsin was observed in this study (data not shown), indicating an increase in resistance to protease caused by γ-irradiation as well as an increase in the degree of cross-linking. Similarly, Liu *et al.* have reported that the introduction of cross-links results in a decreased solubility in PBS and an increased resistance to protease (20). In addition, it was reported that cross-linking leads to an increase in the mechanical strength of collagen (21). These complex changes in the physical properties of γ-irradiated samples might influence cell activities. The effect of γ-irradiation on the physical properties of SAC-EDC samples is under consideration.

The increase in the proliferation rates of MC3T3-E1 cells caused by γ-irradiation saturated at the dose of 15 kGy (Fig. 1), whereas the increase in ALP activities saturated at the dose of 10 kGy (Fig. 2). The reason the dose response of cell activity to

γ-irradiation was different is still unclear. There are some reports that γ-irradiation degrades collagen molecules. Cheung *et al*. have reported that the denaturation level of collagen molecules by γ-irradiation is low (at a dose of less than 10 kGy) and that a progressive decrease in molecular weight is observed at doses greater than 25 kGy (22). In addition, Isama and Tsuchiya have reported that γ-irradiation degrades PLLA molecules and stimulates the differentiation of MC3T3-E1 cells cultured on irradiated PLLA (19). These reports indicate that the degradation of polymers by γ-irradiation is inevitable and that products of the degradation can influence cell activity. In addition, a dose of 10 kGy is less disruptive to the collagen structure than that of 25 kGy. In our study, there were changes in proliferation rates at doses greater than 15 kGy. The results indicate that the degradation or denaturation of SAC by γ-irradiation affects the proliferation rates of MC3T3-E1 cells. We are investigating the degree of the denaturation of γ-irradiated SAC-EDC samples.

In conclusion, in this study, we show that the activities of MC3T3-E1 cells cultured on γ-irradiated scaffolds are changed by γ-irradiation. Generally, a 25 kGy dose of γ-irradiation is recommended for the sterilization of most bioprostheses (22). Before the application of collagen biomaterial in implant devices, it is necessary to consider the changes in cell activity caused by γ-irradiation.

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Figure captions

FIG. 1. Proliferation rates of MC3T3-E1 cells cultured on SAC-EDC scaffolds irradiated at doses of 0 (circles), 10 (squares), 15 (triangles), and 25 (lozenges) kGy, respectively, expressed as MTS absorbance. The bar represents SD (n = 3).

FIG. 2. ALP activities of MC3T3-E1 cells cultured on nonirradiated and irradiated SAC-EDC scaffolds. The bar represents SD (n = 3).

FIG. 3. Degrees of cross-linking (%) of nonirradiated and irradiated SAC-EDC scaffolds, expressed as percentage of primary amine group content lost after cross-linking. The bar represents SD (n = 3).

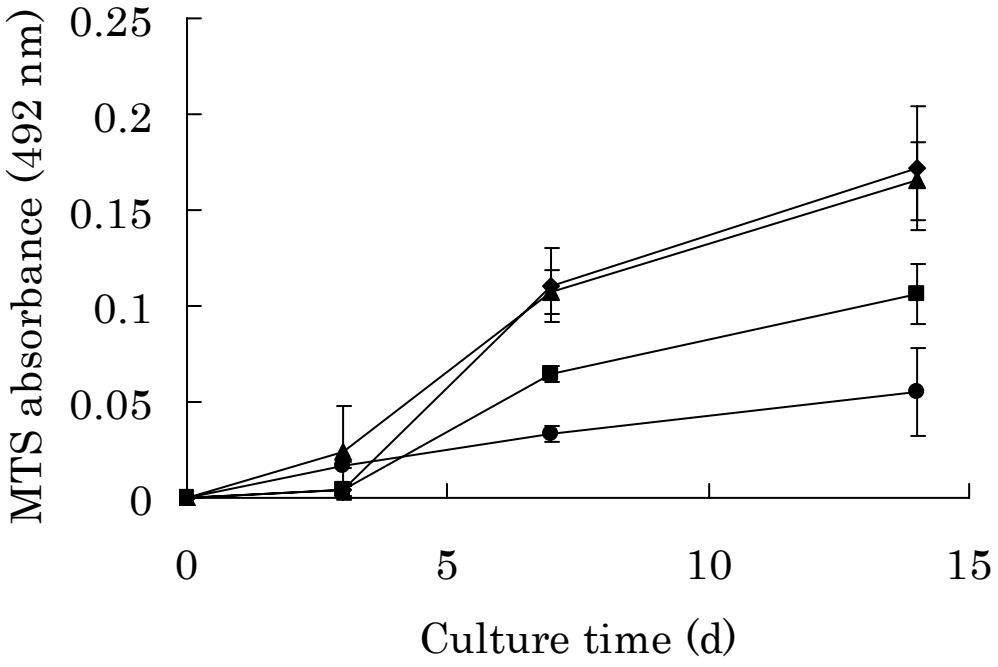
MTS absorbance (492 nm)
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
Culture time (d)

FIG. 1

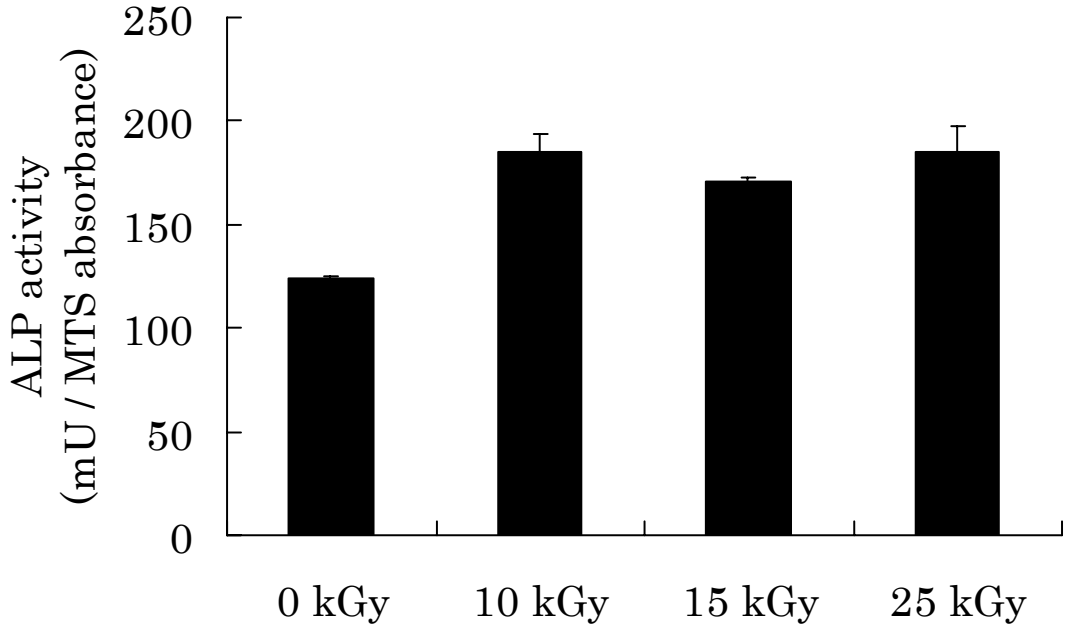
ALP activity
(mU / MTS absorbance)
250
200
150
100
50
0
0 kGy
10 kGy
15 kGy
25 kGy

FIG. 2

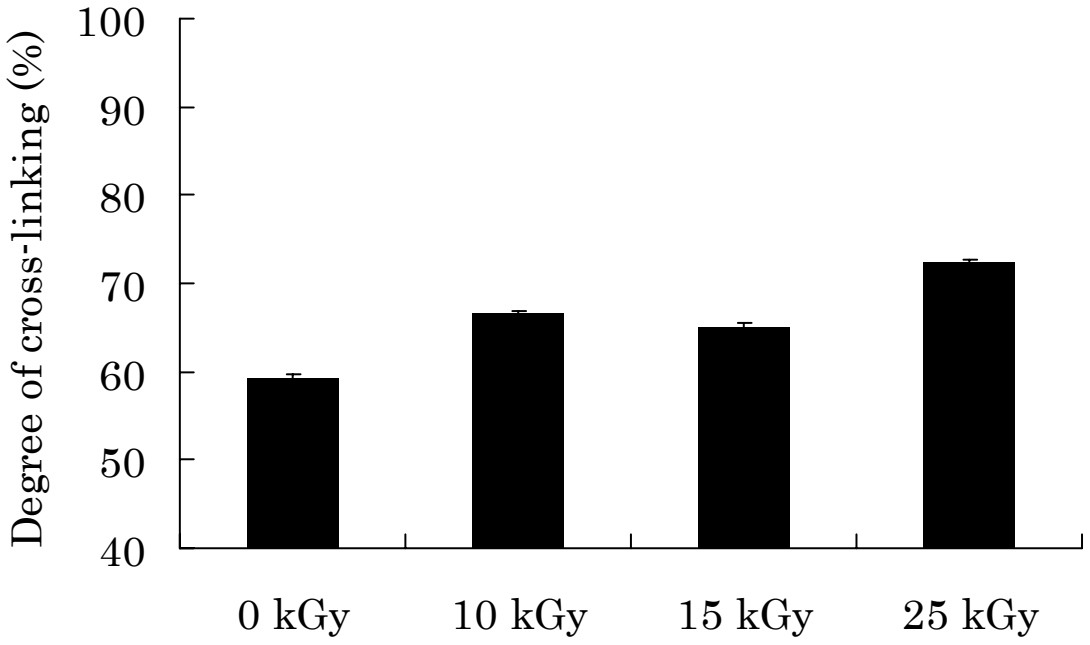
Degree of cross-linking (%)
100
90
80
70
60
50
40
0 kGy
10 kGy
15 kGy
25 kGy

FIG. 3