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Creation of cross-linked electrospun isotypic-elastin fibers controlled cell-differentiation with new cross-linker

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ABSTRACT

Effective application of elastin materials for vascular grafts in tissue engineering requires these materials to retain the elastic and biological properties of native elastin. To clarify the influence of soluble elastin isotypes on vascular smooth muscle cells (VSMCs), soluble elastin was prepared from insoluble elastin by hydrolysis with oxalic acid. Its fractions were separated and classified into three isotypes. Elastin retaining 2.25 mol% of cross-linked structures exhibited significant differentiation of VSMCs, which adhered to the elastin with contraction phenotypes similar to that of native elastin, causing proliferation to cease. This trend was more strongly demonstrated in cotton-like elastin fibers with a new cross-linker. The results suggest that elastin isotypes could be applied as new effective biomaterials for suppressing intimal hyperplasia in vascular grafts.

1. Introduction

In graft-based surgical treatment of vascular diseases such as arteriosclerosis, it is difficult to realize small-diameter vascular grafts without causing intimal hyperplasia and thrombosis that may accompany abnormal reactions to the biomaterial. A technique that mimics the structural and mechanical properties of a native blood vessel would be an important technology that could provide successful results in this scenario [1]. Collagen-based materials have largely been the focus in tissue engineering because they can strongly promote the adhesion of vascular cells [2–4]. In these studies, the inside of a tubular structure such as a mimic vessel was coated with collagen for improving vascular cell adhesion. In addition, collagen-based materials have been promoted for the production of extracellular matrix from proliferating cells. Intimal hyperplasia of vascular grafts is a phenomenon observed in arteriosclerosis; it is considered to be consequence of the phenotypic modulation of vascular smooth muscle cells (VSMCs) [5,6]. Synthesis-type VSMCs promote proliferation and fibrosis of tissues such as collagen-based materials. In contrast, native elastin is known to promote contraction-type VSMCs and to control the differentiation of VSMCs [7–9].

The wall of a blood vessel comprises three layers: Tunica intima, tunica media, and tunica externa. Tunica intima is a thin layer of endothelial cells that are in contact with blood cells and plasma. Tunica media consists of concentrically arranged VSMCs supported by elastin layers, which are connected by submicron-sized elastic fibers [10]. The elastic fibers control the VSMCs for the contractibility of the vessel. Tunica externa is composed of collagen fibers and fibroblasts.

Elastin is an extracellular matrix protein that provides tissue elasticity. Its concentration is particularly high in elastic tissues such as vessels and ligaments [11,12]. The elastic and biological properties of elastin obtained from an organism must be retained for its effective application. However, the use of elastin as a biomaterial has thus far been limited because native elastin is highly insoluble owing to its cross-linked structures. Commercially available soluble elastins such as α-elastin, β-elastin, and κ-elastin obtained from insoluble elastin by hydrolysis are unstable for the differentiation of VSMCs because of the differences in their structures [13–15]. It is known that cross-linking in native elastin is produced by the enzymatic reaction of tropoelastin produced from cells. The cross-linked structures in elastin are desmosine, isodesmosine, and their derivatives [16–18]. The cross-linked structures in insoluble elastin may be degraded by solubility treatment. However, these structures are also considered essential for the structure and function of native elastin.

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Our original hypothesis was that soluble elastin containing cross-linked structures could control phenotypic modulation of VSMCs. Generally, synthetic soluble elastins that can be used as biomaterials, including tropoelastin [19–22] and elastin-like peptides [23–27], do not have the cross-linked structures seen in native elastin. Although elastin extracted from an organism has a high possibility of exhibiting native biological properties for VSMC, the manner in which elastin containing cross-linked structures affects VSMCs is unclear. In addition, because the elastic fibers between the VSMC layers in a blood vessel are thought to be an important factor for cell activation, the formation of fiber structures by elastin is required for the development of biomimetic vascular grafts. Recently, many investigations of fiber preparation by electrospinning with collagen and elastin have been reported [28–30]. However, in these studies, fiber density increased significantly with the accumulation on the collection electrode. The ideal structure of an elastin material is one in which the fiber density is lower and space is available for normal orientation of VSMCs in the native blood vessel. In this paper, we prepared elastin-based material with several kinds of soluble elastin containing cross-linked structures from insoluble elastin. In addition, we investigated the differentiation properties of VSMCs caused by the new soluble elastin isotypes and studied their cotton-like fiber construction.

2. Materials and methods

2.1. Preparation of insoluble elastin

Porcine arteries were obtained from a slaughter center (Matsusaka, Mie, Japan). After removing fat from the arterial tissue (2 kg) with scissors, the tissue was washed in 10 wt% NaCl solution for 24 h at 4 °C. The tissue was cut into 5-mm sections by using a blender; the sections were then heated in an autoclave for 1 h at 121 °C. After washing with deionized water, the sections were maintained in 90 wt% ethanol for 10 h. Subsequent to drying and blending, a dry powder of insoluble elastin (330 g) was obtained.

2.2. Preparation of soluble elastin

Soluble elastin was prepared from insoluble elastin by using the method of Partridge et al. [13,16]. Briefly, insoluble elastin (10 g) was mixed with 0.25 M oxalic acid (45 mL) and maintained at 100 °C for 1 h. The resulting clear yellow solution was cooled at 4 °C and centrifuged at 2000 × *g* for 30 min. The remaining insoluble fraction was mixed with 0.25 M oxalic acid (30 mL) and maintained at 100 °C for 1 h. These procedures were repeated eight times until complete dissolution occurred. The first and second fractions were named elastin-C; the third, fourth, and fifth fractions were named elastin-B; and the sixth fraction and beyond were named elastin-A. Solutions of each of the three classes were dialyzed in deionized water for 7 d by using a cellulose acetate tube (cutting molecular weight = 12–16 kDa; Sanko Junyaku Co., Ltd., Tokyo, Japan) at 4 °C. The soluble elastin powders (total 3.3 g) were obtained by freeze-drying.

2.3. Molecular weight analysis and amino acid analyses

The molecular weight of the soluble elastin was determined by gel permeation chromatography using a TS-gel G3000SW column (Tosoh Corp., Tokyo, Japan). The elution buffer was 0.1 M phosphate buffered saline (PBS, pH 7.0) containing 0.1 M NaCl, and the flow rate was 0.5 mL/h at 10 °C. The applied sample volume was 20 μL (10 mg/mL) and detection was carried out at 280 nm using a UV spectrophotometer (Hitachi, Tokyo, Japan). The molecular weight was determined by comparison with standard markers (1.2–670 kDa; Bio-Rad laboratories, Hercules, CA, USA).

The amino acid compositions of the soluble elastin were analyzed using an automatic amino acid analyzer (JLC/500 V; JEOL, Ltd., Tokyo, Japan). The analyzed samples were prepared by acid hydration with 6 N HCl at 110 °C for 24 h *in vacuo*.

2.4. Concentration of remaining desmosine derivative in soluble elastin

The absorption of 1–5 wt% of the elastin solutions at 270 nm was measured using a UV-160A spectrophotometer (Shimadzu Corp., Kyoto, Japan). From this result, the absorption coefficient ($A_{Elastin}$) of the 1 wt% soluble elastin was calculated. The mole fraction of the desmosine derivatives was calculated by Eq. (1) using the following parameters: commercially available desmosine (EPC, Inc., Saint Charles, MO, USA) (A_{Des} = 68.7), absorbance of tyrosine in 1 wt% elastin solution (A_Y), absorbance of phenylalanine in 1 wt% elastin solution (A_F), absorbance of tryptophan in 1 wt% elastin solution (A_W), and mole concentration (C_m) of 1 wt% soluble elastin. In this measurement, the amount of absorption of the isodesmosine and desmosine derivatives was regarded to be the same.

$$\text{Mole fraction (\%) of desmosine derivative} = \frac{[A_{Elastin} - (A_Y + A_F + A_W)]}{[1000 A_{Des} M_D C_m]} \quad (1)$$

where M_D is the average molecular weight (526.6) of desmosine and isodesmosine as desmosine derivatives, and the absorption coefficients of tyrosine, phenylalanine, and tryptophan were set as 1340, 5500, and 190 ($L\,cm^{-1}\,mol^{-1}$), respectively. The amino acid contents in elastin were obtained by amino acid analysis.

2.5. Coacervation of soluble elastin determined by turbidity

The coacervation of soluble elastin was measured by its turbidity in deionized water. The transmission intensity (T, %) of the sample solution was determined as follows:

$$T(\%) = \frac{I}{I_o} \quad (2)$$

where I is the transmission intensity of the sample solution and I_o is the transmission intensity of the deionized water used as the blank. The transmission intensity was measured by He–Ne laser (Edmund Optics, Barrington, NJ, USA) at 640 nm. Sample solutions of 1 wt% soluble elastin were placed in a quartz cell (path length = 1 cm), and measurements were conducted by varying the temperature from 10 °C to 80 °C. The coacervation temperature was defined as the temperature at which T equals 50%.

2.6. Preparation of cross-linker

We believed that a cross-linker with hydrophobic groups could be used for the production of water-insoluble fiber material from soluble elastin. Because elastin has many hydrophobic domains [31] with elastic properties, the hydrophobic interaction between elastin and the cross-linker was expected to be important in maintaining elasticity. 4-Hydroxyphenyldimethylsulfoniummethylsulfate (DSP)-activated ester [32] was prepared as the cross-linker of soluble elastin as follows. Briefly, 1,12-dodecandicarboxylic acid (Dode, 1 mmol; Sigma–Aldrich Corp., Saint Louis, MO, USA) and DSP (2 mmol) were dissolved in acetonitrile, and dicyclohexylcarbodiimide (DCCD, 2 mmol; Wako Pure Chemical, Osaka, Japan) was then added to the solution at 25 °C while stirring. After stirring for 5 h, dicyclohexyl urea was filtered-out using a G4-glass filter (AGC Techno Glass Co., Chiba, Japan). The cross-linker was recrystallized from diethyl ether at 25 °C and then dried *in vacuo*. Its chemical structure was

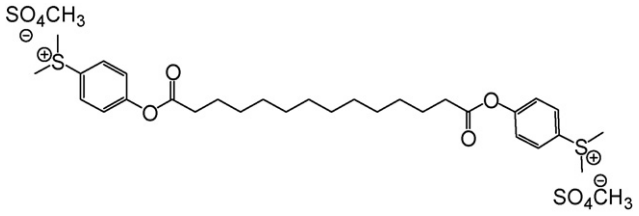

Fig. 1. Chemical structure of the cross-linker – 1,12-dodecanedicarboxylic 4-hydroxyphenyl dimethylsulfonium methylsulfate (Dode–DSP) – used for the production of fiber material from soluble elastin.

identified by 500-MHz ^{1}H NMR (JNM-EX-500; JEOL) (Fig. 1). The Dode–DSP agent was obtained in powder form with 99.2% purity.

2.7. Preparation of cotton-like elastin fiber by electrospinning

We fabricated an original electrospinning device for producing the cotton-like elastin fiber. As shown in Fig. 2, a high-voltage power supply (ES30P-10W/DDPM; Gamma High Voltage Research, Ormond Beach, FL, USA) was attached to the metal nozzle of syringe as an electrode. The syringe was set on a syringe pump (MSP-DT2; As-One Corp., Osaka, Japan) that controlled the flow of elastin solution. The device had a two-type rod structure with a cross-setting that served as a collection chamber for the cotton-like fiber. It also had a negative-charge power supply (Panasonic, Tokyo, Japan) set on the upper section for eliminating electrostatic force. A net

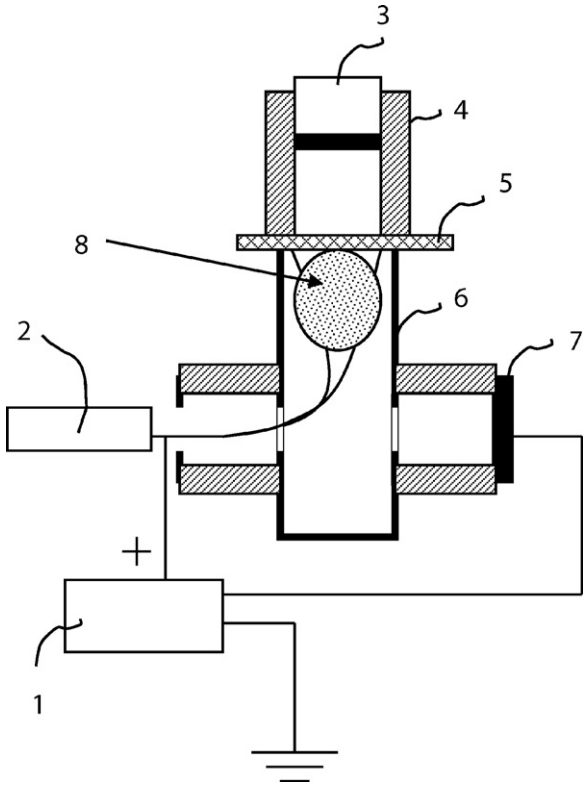

Fig. 2. Schematic of the apparatus for cotton-like fiber construction: 1: high-voltage power supply; 2: syringe pump (for the elution solution); 3: negative-charge power supply; 4: materials to prevent static electricity; 5: collection net; 6: collection chamber; 7: target electrode; and 8: cotton-like fibers of elastin. The elastin solution jet from the nozzle accumulated on the collection electrode (aluminum plate) when the negative-charge power supply was turned off. When it was switched on, the positively charged elastin rose to the upper electrode. During this process, the elastin polymer ion slowed down due to neutralization by the negative-ion shower. The fiber ends then adhered to the collection net, resulting in a chain reaction. Consequently, cotton-like fibers of elastin were formed on the collection net surface.

was installed for collecting the fibers from the collection chamber.

More specifically, 250 mg of soluble elastin (elastin-A) and 20 mg of Dode–DSP were mixed in hexafluoropropanol (HFP, 5 mL; Wako Pure Chemical), and 5 mg of triethylamine (TEA; Wako Pure Chemical) was added to the solution as a catalyst. The flow rate of the elastin solution jet from the syringe was 1.2 mL/h with a voltage of 15 kV. The nozzle of the needle was of G-21 grade stainless steel (Terumo Corp., Tokyo, Japan). The distance between the target electrode and the nozzle was 10 cm. The distance from the negative charge-producing electrode to the target electrode was 15 cm and that to the collection net was 10 cm. As a reference, we produced cotton-like structures without cross-linkers and TEA. The obtained elastin fiber was heated for cross-linking at 121 °C for 20 min.

2.8. Cytotoxicity of soluble elastin and cross-linker for VSMCs

Cytotoxicity was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit-1 (Roche, Basel, Switzerland) to determine the succinate dehydrogenase mitochondrial activity. To determine the cytotoxicity of the soluble elastin and Dode–DSP for VSMCs (Cell Systems Corp., Kirkland, WA, USA), the cells (10 μL) were seeded in 96-well plates (Nalge Nunc International, Tokyo, Japan) at a density of 1.5×10^5 cells/mL at 37 °C in 5% CO_2 atmosphere for 24 h. The medium was 100 μL/well of CS-C medium (Cell Systems) containing 10% fetal bovine serum (FBS). After washing with PBS, CS-C medium containing either soluble elastin (0.1–5 wt%) or Dode–DSP (0.05–0.5 mM) was added to each well for 24 h. After washing with PBS, CS-C medium containing 10 μL of 5 mg/mL MTT stain was added to each well and incubated for 12 h. The absorbance in each well was measured at 550 nm (reference, 655 nm) by using a microplate reader (Model660; Bio-Rad Laboratories).

2.9. Proliferation test of VSMCs for soluble elastin

In the proliferation test, we used a 5-bromo-2′-deoxy-uridine (BrdU) labeling and detection kit III (Roche) for quantitative analysis of the S phase of in the cell cycle; BrdU combined with newly synthesized DNA in the cell. To determine VSMC proliferation by soluble elastin, the cells (10 μL) were seeded in 96-well plates at a density of 2.0×10^5 cells/mL at 37 °C in 5% CO_2 atmosphere for 72 h. After washing with PBS, FBS-free/FBS-containing CS-C medium (100 μL/well) containing soluble elastin (10–200 μg/mL) or coacervated gel was added to each well. After incubating for 6 h with BrdU labeling solutions, anti-BrdU antibody-conjugated peroxidase labeling agent was added to each well at 37 °C for 30 min, and *o*-phenylenediamine (OPD) was then added and measured at 405 nm (reference, 490 nm) by using a microplate reader.

2.10. Differentiation test of VSMCs for soluble elastin

VSMC differentiation induced by soluble elastin was evaluated by performing fluorescence-activated cell sorting (FACS) or western blotting (WB) to measure the amount of smooth muscle (SM) α-actin produced in these cells. VSMCs were cultured under four elastin-forming conditions: solvent, coating, coacervated gel, and cotton-like fiber. To determine the amount of SM α-actin produced, VSMCs in 100 μL/well of CS-C medium were seeded in a 35-mm-diameter cell-culture dish at a density of 1.0×10^5 cells/mL at 37 °C in an atmosphere of 5% CO_2 atmosphere for 3 d.

The cultured cells were subjected to FACS as follows: trypsin solution was added to the dish: the exfoliated cells were centrifuged at 800 rpm for 1 min and immobilized with 70% ethanol for 5 min. To the cell suspension thus obtained, anti-SM α-actin antibody conjugated with fluorescein isothiocyanate (FITC; Dako,

Table 1
Average molecular weights of the soluble elastin isotypes.

	⟨Mw⟩ (kDa)	⟨Mn⟩ (kDa)	Molecular distribution ⟨Mw⟩/⟨Mn⟩
Elastin-A	90.3	25.6	3.5
Elastin-B	52.4	15.3	3.4
Elastin-C	20.0	10.0	2.0

Mw, weight average molecular weight; Mn, number average molecular weight.

Table 3
Mole fraction of the remaining desmosine derivatives in the soluble elastin isotypes (mol%).

	Elastin-A	Elastin-B	Elastin-C
Absorbance coefficient (270 nm, 1 wt%)	10.57	9.57	3.73
Desmosine derivative (mol%)[a]	2.25	1.97	1.04

[a] Total value of desmocine, isodesmocine and related derivatives.

Glostrup, Denmark) was added for 30 min. The intensity of FITC was measured using FACS-Caliber (Becton Dickinson, Franklin Lakes, NJ, USA). Integration of the histogram obtained was calculated as the amount of SM α-actin relative to that of the control.

WB of the cultured cells was performed as follows: the cells were dissolved in 0.1% TritonX-100, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH7.2). Aliquots of each cell lysate (containing equal concentrations of protein) were resolved using 10% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and immunoblotted using anti-SM α-actin antibody with anti-mouse HRP as the secondary antibody.

2.11. Statistical analysis

Data are expressed as mean ±standard deviation (SD). Statistical significance was determined at a P value < 0.05. Calculations were performed using the statistical package StatView 5.0 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Molecular weight of soluble elastin

Table 1 shows the average molecular weight of soluble elastin. The weight average molecular weight (Mw) of elastin-A, elastin-B, and elastin-C was 90.3, 52.4, and 20.0 kDa, respectively. The average molecular weight increased with the number of oxalic acid treatments; the molecular weight distribution of the soluble elastin had a relatively narrow range (Mw/Mn = 2.0–3.5).

3.2. Amino acid content of soluble elastin

Table 2 shows the molarity of the amino acid contents of elastin-A, elastin-B, and elastin-C. The amino acid contents of elastin-A and elastin-B were not significantly different. In addition, the calculated contents of tropoelastin [31] and soluble elastin were comparable, with the exception of lower lysine content. Elastin-C exhibited a slightly higher content of hydrophilic amino acids such as lysine, aspartic acid, and glutamic acid. Because tropoelastin did not have several amino acids such as methionine and histidine, soluble elastin did not contain these amino acids. From the results, all the soluble elastin isotypes were considered to be high purity samples.

3.3. Remaining desmosine derivative concentration in soluble elastin

Desmosine and isodesmosine structures are the cross-linked structures of elastin. In general, the desmosine concentration was determined by high-performance liquid chromatography (HPLC) and was compared with that of the standard sample [33]. However, no desmosine derivative as an incomplete structure destroyed by hydrolysis was detected. Because desmosine and isodesmosine were prepared with four lysine residues in tropoelastin, the theoretical lysine content obtained from the primary structure of tropoelastin (pig) was estimated as 4.2 mol%. However, as shown in Table 2, the lysine concentration of elastin-A was calculated to be 0.8 mol%, which was very low; in addition, other amino acid contents were almost the same as that in tropoelastin. These results demonstrated that the low content of lysine could be caused by changes in desmosine derivatives. If the desmosine derivatives in elastin could be measured accurately, the method would be suitable for the measurement of the remaining cross-linked structures.

In Table 3, we show the determination of the desmosine derivative concentration using Eq. (1) from spectroscopic analysis. The absorption coefficients of elastin-A, elastin-B, and elastin-C were 10.57, 9.57, and 3.73, respectively. The desmosine derivative concentrations were determined by these values, and the concentrations of elastin-A, elastin-B, and elastin-C were calculated to be 2.25, 1.97, and 1.04 mol%, respectively. These results clarified that the remain-

Table 2
Amino acid contents of the soluble elastin isotypes (mole fraction).

Amino acids	Mol fraction (mol%)				
	Elastin-A	Elastin-B	Elastin-C	Tropoelastin (pig) Ref. [31]	Tropoelastin (human) Ref. [31]
Glycine	32.4	33.3	31.1	32.3	30.4
Alanine	28.6	23.3	17.9	24.7	28.2
Valine	13.3	15.5	12.9	14.0	12.4
Proline	4.4	4.7	4.9	10.9	11.0
Phenylalanine	3.2	3.2	3.2	2.8	4.1
Leucine	6.3	6.0	6.1	4.9	4.9
Isoleucine	1.7	2.0	2.4	1.6	2.1
Serine	1.4	1.6	2.8	0.6	1.4
Threonine	1.7	1.7	2.5	1.0	0.03
Tyrosine	2.5	1.6	1.6	1.5	0.6
Lysine	0.8	1.1	2.2	4.2	3.5
Arginine	0.6	0.9	2.1	0.3	0.01
Aspartic acid +Asparagine	0.3	1.3	3.1	0.0	0.0
Glutaminic acid +Glutamine	2.6	3.2	5.7	1.1	2.0
Histidine	0.3	0.5	1.1	0.0	0.0
Methionine	0.0	0.1	0.3	0.0	0.0
Total	100.0	100.0	100.0	100.0	100.0

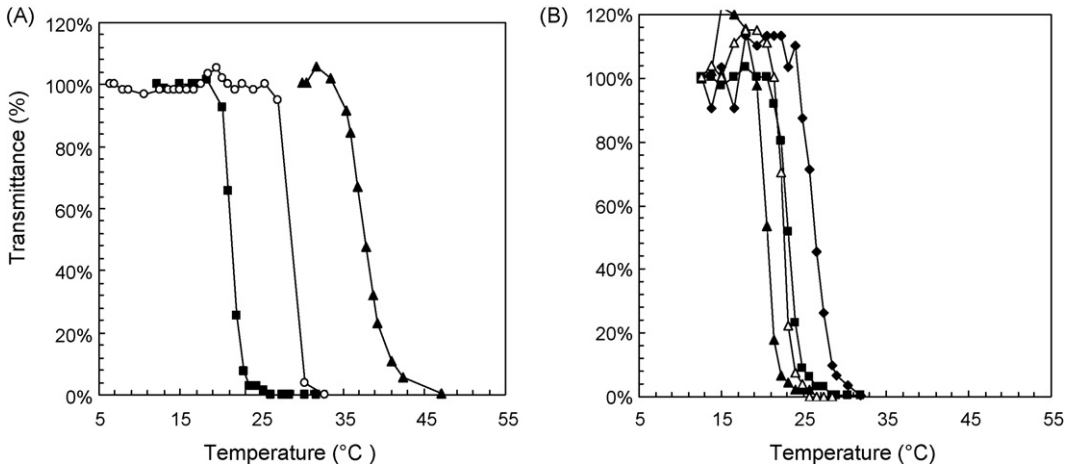

Fig. 3. Coacervation properties of the soluble elastin isotypes. (A) (■) Elastin-A, (○) elastin-B, and (▲) elastin-C. (B) (▲) Elastin-A:elastin-C = 8:2, (■) elastin-A:elastin-C = 5:5, (♦) elastin-A:elastin-C = 2:8 (1 wt% elastin), and (△) elastin-A:elastin-C = 5:5 (2 wt% elastin).

ing concentration of the desmosine derivatives increased with the number of oxalic acid treatments (hydration).

3.4. Coacervation temperature of soluble elastin

The elastin solutions were observed to coacervate at a characteristic temperature, a phenomenon known as thermoreversible aggregation by hydrophobic interaction of molecules. Fig. 3A shows the coacervation properties of the soluble elastin isotypes. The coacervation temperatures of 1 wt% elastin-A, elastin-B, and elastin-C solutions were 21.3 °C, 28.7 °C, and 37.6 °C, respectively. Lower coacervation temperature indicated improved aggregation by intermolecular interaction. Fig. 3B shows the behavior of a mixed solution comprising two isotypes of soluble elastin. In the case of the solution with elastin-A and elastin-C, the coacervation temperatures of 1 wt% elastin-A:elastin-C at concentrations of 8:2, 5:5, and 2:8 were 21.4 °C, 23.0 °C, and 26.0 °C, respectively. Coacervation behavior, which was similar to that for an unmixed solution was determined by the compositions of the two components without considering the total concentration, because the data for 1 wt% (elastin-A:elastin-C = 5:5) and 2 wt% were obtained at the same temperature (23.0 °C and 22.7 °C).

3.5. Preparation of cotton-like structure

Fig. 4A and B shows the cotton-like structure produced by our improved electrospinning apparatus (Fig. 2). The density of the fibers was 5–10 mg/cm^3 with a cotton-like appearance, and the diameter of the fibers was about 1–10 μm (average 5.1 ± 1.4 μm) in dry condition. This fiber matrix was heated for 20 min at 121 °C to enable cross-linking and was prepared under several conditions with a cross-linker and by catalysis. The water solubility of the cotton-like fibers was observed in deionized water for 10 min at 37 °C. The fiber material produced using a cross-linker and cata-

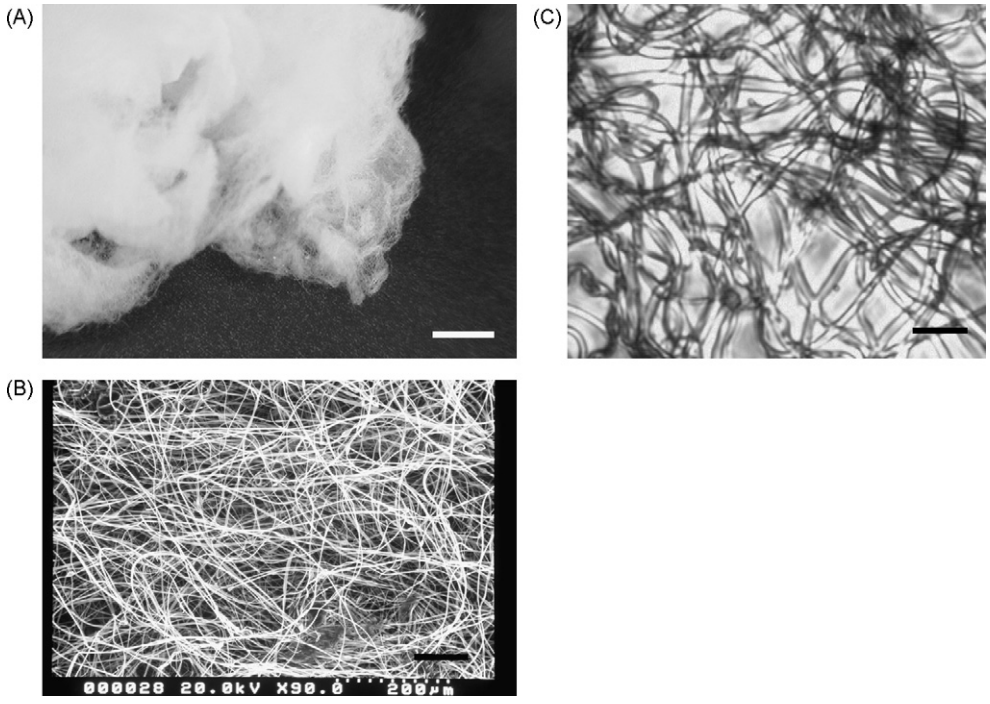

Fig. 4. Cotton-like fiber material of elastin-A. (A) Elastin fiber in dry condition (scale bar = 1 cm), (B) elastin fiber in dry condition (scale bar = 100 μm), and (C) elastin fiber in wet condition in medium (scale bar = 100 μm).

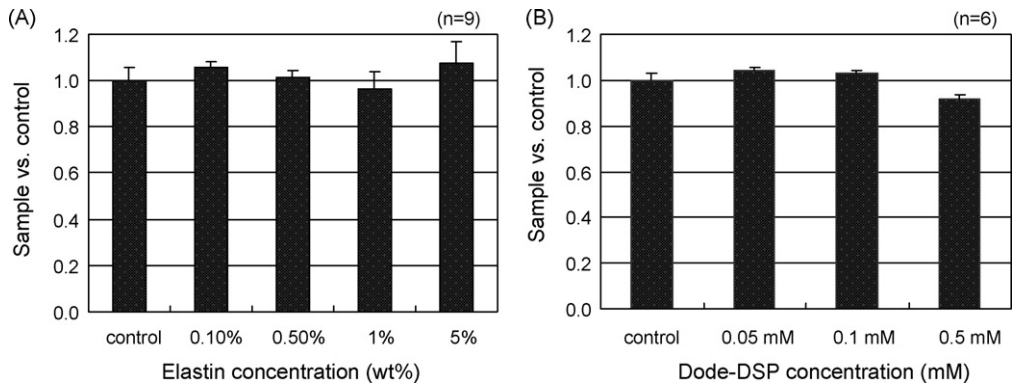

Fig. 5. Cytotoxicity in vascular smooth muscle cells (VSMCs) for soluble elastin and the cross-linker measured by the MTT test. (A) Elastin-A and (B) 1,12-dodecanedicarboxylic 4-hydroxyphenyl dimethylsulfonium methylsulfate (Dode–DSP) were added to the cell culture medium and cultured for 24 h. The measurement values were normalized to that of the control condition.

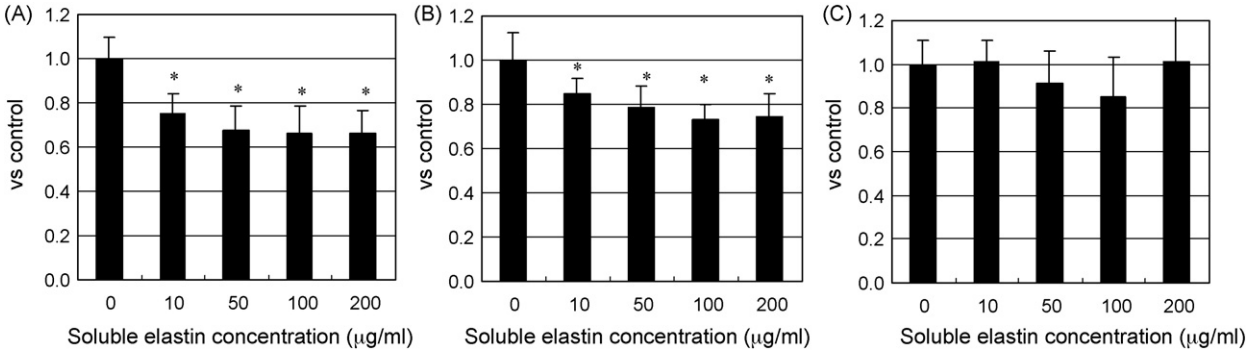

Fig. 6. Proliferation of vascular smooth muscle cells (VSMCs) with fetal bovine serum (FBS)-free medium measured by the BrdU test. (A) Elastin-A, (B) elastin-B, and (C) elastin-C were added to the cell culture medium and cultured for 72 h. BrdU was added into the medium (FBS-free) for 6 h and measured at an absorbance of 405 nm (reference 490 nm). The measured values were normalized to that of the control condition: $^{*}P < 0.05$ ($n = 10$).

lyst was the only one insoluble in water, whereas fibers prepared under other conditions were soluble. The cross-linked fibers produced from elastin-A are shown in the wet condition in Fig. 4C. The swollen fiber diameter was 10–30 μm (average 17.5 ± 4.1 μm), and this fiber contained flexible elastin gel with the capability to elongate by 200%. On the other hand, the cotton-like fibers produced from elastin-B and elastin-C did not react with Dode–DSP. These fibers dissolved in water even when using both the cross-linker and catalyst.

3.6. Cytotoxicity of VSMCs for soluble elastin and cross-linker

The cytotoxicity of elastin-A and Dode–DSP for VSMCs was estimated by the MTT test, and the results are shown in Fig. 5A and B, respectively. Because the cytotoxicities of elastin-A and Dode–DSP were similar in the control condition, it was concluded that these molecules did not exhibit cytotoxicity. In addition, DSP alone did not exhibit cytotoxicity toward VSMCs (data not shown).

3.7. Proliferation (BrdU) test of VSMCs for elastin materials

The BrdU test is considered to be a proliferation test based on the correlation between cell proliferation and BrdU absorption. The relative results of the BrdU test in the control condition are shown in Fig. 6. In the condition in which the medium contained 200 μg/mL of elastin-A, proliferation of VSMCs was suppressed up to 67.0% ± 10% compared to the control. Similarly, proliferation in the elastin-B-containing medium was suppressed up to 74.0% ± 10%, but proliferation in elastin-C-containing medium was not suppressed. In the case of the FBS-containing medium, the results of the BrdU test with respect to the control conditions are shown in Fig. 7. In this case, proliferation of VSMCs in the elastin-A-containing medium and elastin-B-containing medium was suppressed up to 70.0% ± 22% and 74.8% ± 26%, respectively. Proliferation in the elastin-C-containing medium

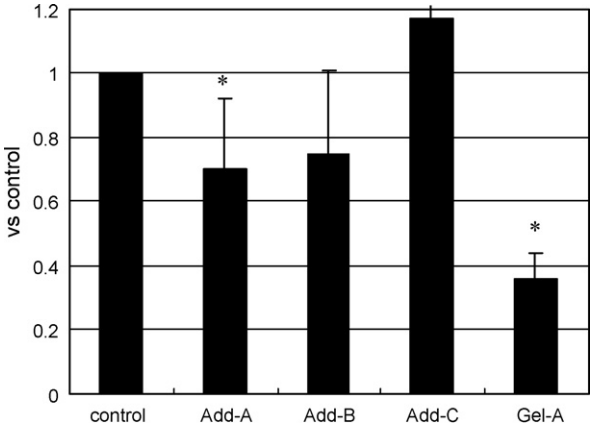

Fig. 7. Proliferation of vascular smooth muscle cells (VSMCs) with medium containing fetal bovine serum (FBS), measured by the BrdU test. Elastin-A (Add-A) elastin-B (Add-B) and elastin-C (Add-C) (100 μg/mL each) were added to the cell culture medium containing 10% FBS and cultured for 72 h. Gel-A: coacervated gel of elastin-A covering VSMCs. The coacervated gel formed by elastin-A (750 mg/mL) covered the cells at 37 °C for 72 h, and the thickness of the gel was about 100 μ m. The measured values were normalized to that of the control condition: $^{*}P < 0.05$ ($n = 10$).

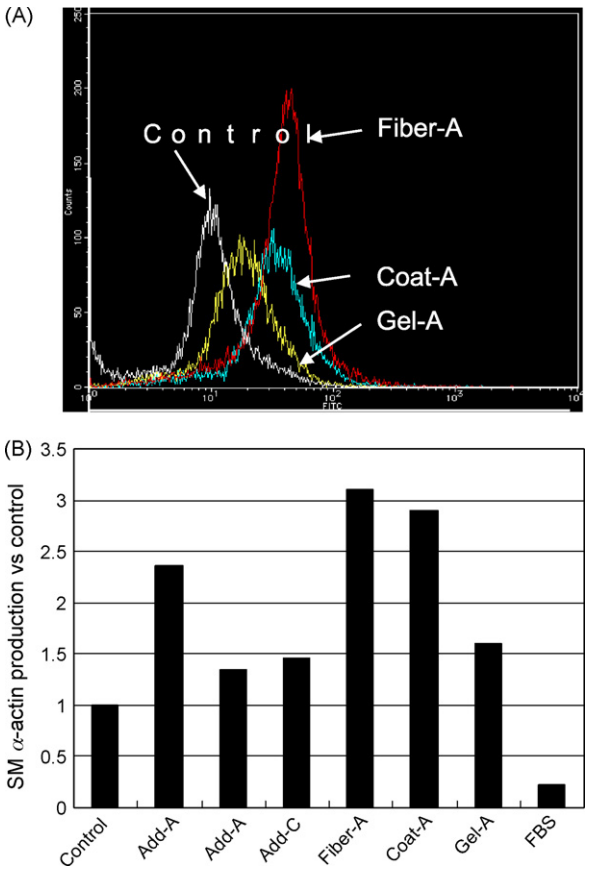

Fig. 8. Production of smooth muscle (SM) α-actin under different conditions. (A) fluorescence-activated cell sorting (FACS) data and (B) the relative values versus that in the control condition obtained by accumulation of fluorescence intensity. Proliferation of vascular smooth muscle cells (VSMCs) in the medium containing 10% fetal bovine serum (FBS): 100 μg/mL of elastin-A (Add-A), elastin-B (Add-B) and elastin-C (Add-C) were added to the cell culture medium. Fiber-A: cotton-like fiber (10 mg/cm^3) of elastin-A, Coat-A: coating by elastin-A, and Gel-A: coacervated gel of elastin-A covering VSMCs. The medium used was 100 μL/well of CS-C medium containing 10% FBS. For the solvent condition, a final elastin-A concentration of 100 μg/mL was used. For the coating condition, the surface of the suspended cell culture dish was activated by corona discharge with a corona Fit CFG-500 (Shinko Electric & Instrumentation Corp., Osaka, Japan) at 9 kV for 45 s. Then, 1 mL of elastin-A solution (100 μg/mL) was added into the dish for covalent bonding on the activated surface of the dish (made of polystyrene) for 8 h at 4 °C.

was not suppressed, similar to the nonserum condition. In the coacervated gel (Gel-A) formation condition, proliferation in the elastin-A-containing medium was remarkably suppressed up to 35.8% ± 8.3%.

3.8. Differentiation (FACS and WB) tests of VSMCs for elastin materials

SM α-actin production from VSMCs under conditions of elastin-A addition, elastin-A coating, cotton-like fibers addition, and coacervated gel formation was determined in comparison with that from nontreated cells under control conditions for 3 d. The results are shown in Fig. 8. In the case of elastin-A addition, SM α-actin production increased 2.4 times over that in the control condition. With an elastin-A coating, SM α-actin production increased 2.9 times. Production increased 3.1 times with the addition of cotton-like fibers. VSMC differentiation clearly accelerated with the development of the cytoskeleton in the presemce of SM α-actin. The surface of the cotton-like fibers resembled that of the dish coated with elastin-A. The amount of SM α-actin, produced in the presence of coacervated gel was 1.6 times that in the control condition.

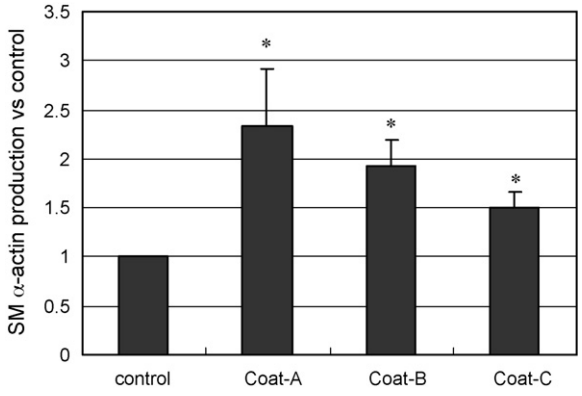

Fig. 9. Production of smooth muscle (SM) α-actin under solid-phase conditions. Relative values versus the values in the control condition, as determined using the absorbance at 405 nm and western blotting (WB). Proliferation of vascular smooth muscle cells (VSMCs) in a medium containing 10% fetal bovine serum (FBS): Coat-A: coating by elastin-A, Coat-B: coating by elastin-B, and Coat-C: coating by elastin-C. The measured values were normalized to that of the control condition: $^*P < 0.05$ ($n = 3$).

Because VSMCs in the medium containing FBS produced 0.2 times more SM α-actin, we concluded that a state of dedifferentiation prevailed.

Fig. 9 shows the results of WB for SM α-actin produced under solid-phase conditions in the presence of an elastin coating. With the elastin-A coating, SM α-actin production increased 2.3 ± 0.6 times over that in the control condition. Further, the amount of SM α-actin produced in the presence of the elastin-A coating, as determined using FACS, was identical to the amount determined using WB. In the presence of elastin-B and elastin-C coating, SM α-actin production decreased 1.9 ± 0.3 and 1.5 ± 0.2 times, respectively. The interaction between the cells and the elastin substrate as the solid phase was much more effective for VSMC differentiation than the addition of soluble elastin to the culture medium.

4. Discussion

4.1. Elastin materials using soluble elastin isotypes

To date, analysis of the purity of soluble elastin extracted from organs [15], has been difficult due to the degradation of the mixture. The main reason for this difficulty is considered to be the unknown higher-order structure of elastin containing cross-linked structures. In this study, we successfully prepared a soluble elastin with remaining cross-linked structures and clearly demonstrated a higher-order structure, as shown by the results given in Table 3. Desmosine or isodesmosine as the hydrophobic cross-linked structure in native elastin was prepared from four allysine or lysine residues oxidized by a lysil oxidase enzyme with a special sequence of KAAK or KAAAK [12,33]. Generally, because insoluble elastin obtained from organs has cross-linked structures, it is not soluble in water or organic solvents. It is well known that the soluble elastin molecule prepared by hydrolysis is a mixture of several elastin molecules containing the remaining cross-linked structures. Soluble elastin is known to exhibit coacervation by a liquid–liquid phase separation. This coacervation is reversible by the effect of hydrophobic intermolecular interactions and the unique chemical structures of elastin [34,35]. The mixture solution of soluble elastin has only one coacervation temperature, which is the average value for all the molecules. Therefore, classification by the difference in coacervation temperature from the hydrolysis mixture is very difficult. In Fig. 3B, which shows the condition in which elastin-A and elastin-C were mixed, the coacervation temperature was determined as the average of the two individual temperatures. Core

formation of aggregation is considered to occur with the multiplier effect of each elastin molecule; the elastin solution condition is thought to exist only at the phase-transition temperature. This effect increases remarkably with an increase in elastin concentration. Therefore, it is difficult to purify elastin from the hydrolysis mixture by employing coacervation conditions alone.

In this study, we obtained hydrolysis-grade elastin from insoluble elastin by using phased hydrolysis with oxalic acid. Because both easily degradable and difficult-to-degrade fractions obtained on hydrolysis with oxalic acid were collected by separation, several fractions of soluble elastin containing the remaining cross-linked structures and high-molecular-weight compounds were successfully obtained. The non-degradable fraction obtained from on the hydrolysis of insoluble elastin contained high-molecular-weight elastin (elastin-A) along with some cross-linked structures. The concentration of desmosine derivatives in soluble elastin was 0.0–4.2 mol%, as calculated on the basis of the lysine contents in tropoelastin and by Eq. (1). This value would have been 0–1 mol% if all cross-links with inner molecules of elastin were formed. A value of 1–4.2 mol% is thought to signify a linkage between the inner and inter molecules, which exists as branches of the cross-linked structure. In Table 3, we show that the soluble elastin fractions were prepared with 1.04–2.25 mol% of desmosine derivatives by collecting the products obtained by degradation with oxalic acid. From Figs. 6–9, it is apparent that the soluble elastin fractions (elastin-A and elastin-B) promoted the differentiation and suppression of VSMC proliferation. In constant, the other fraction (elastin-C) exhibited reverse properties.

Figs. 8 and 9 show that the addition of fiber-A (fiber-B and C did not form), and coat-A, B and C to the culture medium was much more effective for inducing VSMC differentiation than the addition of soluble elastin (add-A, B and C). IN particular, the interaction between VSMC and elastin-A as the solid phase was most effective for VSMC differentiation. In the contraction-type of VSMC promoted SM α-actin production, the stress-fiber formation by SM α-actin in the cells is considered to occur through focal adhesion (FA) and contact with the extracellular matrix. In addition, when VSMCs were arranged on fibers (fiber-A) with low density, such as cotton, the VSMCs differentiated along the shape of the fibers. These results have important implications for the effective processing of biomaterials with different isotypes of elastin.

Selection of a suitable soluble elastin for use of as a vascular graft material is very important in tissue engineering. Elastin-A, which contains the remaining desmosine derivatives, has a low coacervation temperature, i.e., of 21 °C at 1 wt%. Generally, α-elastin produced on hydrolysis with oxalic acid is thought to have the same coacervation properties as other types of elastin. The coacervation of soluble elastin (α-elastin) has been extensively investigated and is believed to occur because of the physicochemical changes associated with the interaction between the higher-order and hydrophobic structures of elastin [26,35]. The coacervation temperature changes with the addition of ions or solvents, and the aggregation properties of elastin are considered to be important factors in the preparation of elastin biomaterials. We prepared elastin by using (1) the amino group of a remaining cross-linker as the reaction point and (2) a hydrophobic cross-linker (Dode–DSP) to increase the elastic modulus by hydrophobic interaction. It is important to elucidate the remaining entire cross-link structure of elastin-A to resolve the problems related to intimal hyperplasia.

A contraction-type VSMC phenotype that stops proliferation could be applied for curing arteriosclerosis. This strategy is very important to prevent intimal hyperplasia during tissue engineering of a vascular graft. Recently, it was clarified that proliferation-type VSMCs exhibiting dedifferentiation showed transformation of differentiation by elastin [9]. The interaction of the elastin receptor on the cell surface with elastin promotes signal transduction of the cell skeletal structure. However, an appropriate receptor for elastin remains to be found, except for a few reports that have suggested elastin-binding protein (EBP) [36–38] and integrin αvβ3 as options [39]. All types of soluble elastin do not exhibit similar properties of interactions in VSMCs. The VSMC layer of an artery has an elastic fiber matrix, and cell layers are sandwiched between the elastic layers [10]. From our results (Figs. 8 and 9), we demonstrated that soluble elastin containing native cross-linked structures promoted the cessation of VSMC proliferation by phenotypic modulation. We believe that abnormal proliferation in arteriosclerosis could be suppressed by using a native elastin such as elastin-A.

The proliferation properties of VSMCs were promoted in the cell cycle by the addition of FBS, and the promotion was accelerated with proliferation. Elastin-A suppressed the proliferation of VSMCs even with the addition of FBS (Fig. 7). The origins of signals from the elastin receptor to VSMCs are considered to lie in both the signal transduction with G-protein through the receptor on the surface of VSMCs and the guidance of contraction-type VSMCs during development of actin filament structures in these cells [9]. In particular, this reaction was considered to be an interaction between EBP as the receptor in VSMCs and the VGVAPG hexapeptide order of elastin [40]. However, another report described the lower cooperation of VGVAPG with the elastin–laminin receptor (ELR) [38] known as EBP. In addition, the mechanically activated receptor on VSMCs can respond to changes in blood pressure. We believe that the signal transduction for phenotypic modulation is controlled by cross-linked structures of native elastin because a native cross-linked structure is three-dimensional and covered with effective parts of tropoelastin such as VGVAPG.

These results will be beneficial for the preparation of mimetic media by tissue engineering. The elastin fiber matrix with a cotton-like structure has significant potential in biomaterial investigation. The proliferation suppression effect for VSMCs by the cotton-like fiber matrix that mimicked native media was stronger than the elastin-coating material only. Our results are the first to provide information on controlling VSMC differentiation by using a cotton-like fiber material with elastin-A. We consider this property to be beneficial for the use of elastin as a biomaterial and graft construction material and for the suppression of intimal hyperplasia in the development in small-diameter vascular grafts.

5. Conclusions

We successfully prepared elastin isotypes containing cross-linked structures from a native blood vessel. The new soluble elastin fractions have properties that effectively suppress VSMC proliferation. Elastin-A with 2.25 mol% of the remaining cross-linked structures exhibits the strongest differentiation effect for VSMCs, which adhered to elastin-A as a contraction phenotype and caused the cessation of proliferation. This trend was strongly demonstrated in the cotton-like elastin fibers of elastin-A.

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