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Elastin peptides prepared from piscine and mammalian elastic tissues inhibit collagen-induced platelet aggregation and stimulate migration and proliferation of human skin fibroblasts

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We obtained pure elastin peptides from bovine ligamentum nuchae, porcine aorta, and bonito bulbus arteriosus. The inhibitory activity of these elastin peptides on platelet aggregation induced by collagen and the migratory and proliferative responsivenesses of human skin fibroblasts to these elastin peptides were examined. All of bonito, bovine, and porcine elastin peptides found to inhibit platelet aggregation, but bonito elastin peptides showed a higher inhibitory activity than bovine and porcine elastin peptides did. All elastin peptides enhanced the proliferation of fibroblasts 3.5- to 4.5-fold at a concentration of 10 μg/ml. Bovine and porcine elastin peptides stimulated the migration of fibroblasts, with the optimal response occurring at 10^{-1} μg/ml, while maximal response was at 10^{2} μg/ml for bonito elastin peptides. Furthermore, pretreatment of fibroblasts by lactose depressed their ability to migrate in response to all elastin peptides, suggesting the involvement of elastin receptor in cell response. These results suggest that both mammalian and piscine elastin peptides can be applied as useful biomaterials in which elasticity, antithrombotic property, and the enhancement of cell migration and proliferation are required. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: elastin peptides; bonito bulbus arteriosus; porcine aorta; bovine ligamentum nuchae; platelet aggregation inhibition; fibroblast proliferation; fibroblast migration

Introduction

Elastin is the core protein of elastic fibers in the elastic tissues of most vertebrates. Elastin is abundant in tissues of large arteries and plays an essential role in tissue biomechanics, providing the extensibility and recoil. Elastin is remarkably stable in healthy tissue, with an estimated half-life of 70 years [1], making it the most persistent protein in human body. This, together with its stability due to specific interchain crosslinks, makes elastin a desirable protein for tissue engineering. However, elastin has found little use as a biomaterial [2–4]. The development of durable synthetic vascular grafts has been limited by surface-induced thrombus formation. For this reason, we paid attention to inhibition of platelet aggregation. Ordinas *et al*. [5] and Refelson *et al*. [6] indicated that there is an unambiguous interaction between platelets and elastin fibers and Kabemba *et al*. [7] found that the elastin layer of the vascular wall has an antithrombotic action. Moreover, Sekiya and Okuda [8] reported that elastin peptides prepared from bovine ligamentum nuchae inhibit platelet aggregation induced by thrombin. From this finding and the fact that major constituents of vascular wall include elastin and collagen, a further study was done to explore whether elastin peptides from species other than bovine inhibit collagen-induced platelet aggregation.

It is of interest that elastin peptides are applied to biomaterials like skin substitutes. For this purpose, it is important to examine whether elastin peptides are capable of regulating proliferation and migration of fibroblasts. Fibroblasts are a major dermal component and known to interact with elastin. Thus, a variety of chemoattractants for fibroblasts have been identified including fibronectin, elastin peptides prepared from human aorta and bovine ligamentum nuchae, and synthetic peptides of elastin [9–14]. However, there is no information available on the chemotactic activity of porcine and piscine elastin peptides. Furthermore, it was reported that synthetic peptides of elastin induce the proliferation of fibroblasts [15], but so far little

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information on whether bovine, porcine, and piscine elastin peptides stimulate the proliferation of fibroblasts are available.

There have been misgivings about safety for biomaterial application of elastin peptides from mammalians because of complications like bovine spongiform encephalopathy. For this reason, we chose fish, especially bonitos instead of mammalia. Bonitos are caught in large quantities in Japan and their bulbus arteriosuses are enriched in elastin and are easily available.

In this study, we prepared elastin peptides from bovine ligamentum nuchae, porcine aorta, and bonito bulbus arteriosus and examined the inhibitory activity of these elastin peptides on platelet aggregation induced by collagen and the responses of migration and proliferation of human skin fibroblasts to these elastin peptides.

Materials and Methods

Materials

Bonito bulbus arteriosus was provided by Hayashikane Sangyo Co., Ltd., Shimonoseki, Japan. Bovine ligamentum nuchae was from Kagoshima Federation of Economic Agricultural Cooperative Associations, Kagoshima, Japan, and porcine aorta was from Kyushu Kyodoh Syokuniku Co., Ltd., Chikushino, Fukuoka, Japan. Collagen, MCM Collagen H from horse tendon, was purchased from MC Medical, Inc., Tokyo, Japan. Human skin fibroblasts were from Sanko Junyaku Co., Ltd., Tokyo, Japan. Fibronectin from human foreskin fibroblasts was from Sigma Chemical Co., Ltd., St. Louis, and 2-*o*-tetradecanoylphorbol 13-acetate (TPA) was from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Preparation of Elastin Peptides from Bonito Bulbus Arteriosus

Bonito bulbus arteriosus, which was cut into pieces of about 0.5 cm^3, was defatted by the method of Nakaba *et al*. [16]. Defatted tissues were minced to a fine powder and gently stirred with 10 volume (10 ml/g) 1 M NaCl at 4 °C for 24 h. After extraction, the pellet was recovered by centrifugation at 7000 *g* for 20 min at 4 °C. The same treatment was repeated six times. After the last extraction step, the pellet was recovered by centrifugation at 7000 *g* for 20 min at 4 °C, washed with demineralized water, dried, and then suspended subsequently in 10 volume 0.05N NaOH at 50 °C for 30 min, 10 volume 0.05N NaOH at 50 °C for 15 min (twice), and 10 volume 0.01 N NaOH at 4 °C for 15 min (three times). After the last extraction step, the pellet was recovered by centrifugation at 7000 *g* for 20 min at 4 °C, washed, and dried. The dried product, which was a highly purified insoluble elastin, was suspended in 10 volume 0.25 M oxalic acid for 30 min at 100 °C (four times). The supernatant containing fragmented peptides of insoluble elastin was recovered by centrifugation at 8000 *g* for 20 min at 4 °C, dialyzed in water, and lyophilized.

Preparation of Elastin Peptides from Bovine Ligamentum Nuchae and Porcine Aorta

Bovine ligamentum nuchae or porcine aorta, which was cut into pieces of about 0.5 cm^3, was defatted with 5 volume acetone at 4 °C for 4 h (three times). Defatted tissues were minced to a fine powder and gently stirred with 10 volume 1 M NaCl at 4 °C for 24 h. After extraction, the pellet was recovered by centrifugation at 7000 *g* for 20 min at 4 °C. The same treatment was repeated five times. After the last extraction step, the pellet was recovered by centrifugation at 7000 *g* for 20 min at 4 °C, washed with demineralized water, dried, and then suspended in 10 volume 0.1 N NaOH at 100 °C for 15 min (five times). After the last extraction step, the pellet, which was a highly purified insoluble elastin, was recovered by centrifugation at 7000 *g* for 20 min at 4 °C, washed with demineralized water, dried, and then suspended in 30 volume 0.25 M oxalic acid for 1 h at 100 °C (six times). The supernatant containing fragmented peptides of insoluble elastin was recovered by centrifugation at 8000 *g* for 20 min at 4 °C, dialyzed in water, and lyophilized.

Gel Filtration Chromatography

Bonito, bovine, and porcine elastin peptides were applied to HPLC on a TSK gel G3000 SW_{XL} column (0.78 × 60 cm^2) and eluted with 0.1 M phosphate buffer containing 0.5% SDS (pH 7.5) at a flow rate of 0.5 ml/min. Thyroglobulin (669 kDa), catalase (232 kDa), L-lactate dehydrogenase (140 kDa), and bovine serum albumin (66 kDa) were used as molecular mass standards.

Amino Acid Analysis

Each of bonito, bovine, and porcine elastin peptides was hydrolyzed under vacuum with 6 M HCl for 48 h at 110 °C, dried, and dissolved in 0.02 M HCl. Amino acid analysis was carried out on a JLC-500/V model (Jeol Ltd. Tokyo, Japan), using lithium buffers with increasing pH.

Preparation of Platelets

Human blood was collected in tubes with 0.1 volume 3.2% sodium citrate as an anticoagulant. The blood was centrifuged at 300 *g* for 10 min at 37 °C and the upper layer of platelet-rich plasma was transferred to plastic tubes. Platelet-poor plasma was prepared by centrifuging platelet-rich plasma at 600 *g* for 15 min at 37 °C. Platelet-rich plasma and platelet-poor plasma obtained were used for aggregation test.

Measurement of Inhibitory Activity by Elastin Peptides on Platelet Aggregation

Platelet aggregation was measured by the method of Born [17]. Transmission (%) was calibrated as 0% for platelet-rich plasma and 100% for platelet-poor plasma. Various concentrations of bonito, bovine, and porcine elastin peptides at 0, 0.19, 0.36, 0.52, 1.9, 3.6, 5.2, 7.5, and 20.9 mg/ml were prepared by using platelet-poor plasma. Each of these elastin peptides was added to 0.25 ml of platelet-rich plasma (5 × 10^3 platelets/μl) stirring at a constant speed of 1200 rpm at 37 °C before adding 7 μl of 0.5 mg/ml collagen. The concentration of collagen added was the minimum concentration to obtain the maximum aggregation. Inhibition of platelet aggregation was expressed as a decrease in transmission (%) at 360 nm.

Cell Culture

Human skin fibroblasts were cultured using a routine explants method. Briefly, the cells were plated at a density of 2.2 × 10^5 cells/cm^2 in 35 or 100 mm diameter dishes (Falcon) and grown to preconfluent density in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 in air at 37 °C. Cells at 3–6 passages were used in the following experiments.

Cell Proliferation

Cultures of preconfluent fibroblasts were placed in 0.5% FBS/DMEM for 48 h to bring the cells to a quiescent state and then treated with TPA (positive control) and various concentrations of bonito, bovine, and porcine elastin peptides for 48 h. Cell proliferation was expressed by measuring the absorbance at 490 nm using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan).

Cell Migration

Cell migration was performed using a 48-well microchemotaxis chamber [18]. Briefly, the lower compartment of the chamber was charged with 27 μl of each of bonito, bovine, and porcine elastin peptides at a concentration gradient ranging from 10^{-4} to 10^{4} μg/ml. A polycarbonate filter with 8-μm pore size was used to separate the upper and lower compartments. An aliquot (50 μl) of a fibroblast suspension (1×10^{6} cells/ml) in DMEM was added to the upper compartment. After a 6-h incubation at 37 °C in a humidified atmosphere of 5% CO_2 in air, the filter was removed from the chamber, fixed in methanol, and stained with Diff-Quick dye. Cell migration was expressed as the net number of cells that migrated through the 8-μm pores in five random high-power fields (×400) for each of triplicate filters. Fibronectin was a positive control and DMEM was a negative control.

Lactose is known to be an antagonist of elastin receptor. A 67-kDa elastin binding protein, the peripheral membrane protein subunit of elastin receptor, contains two binding sites: one for elastin and the other for lectin [19]. The association between elastin and the 67-kDa elastin binding protein is allosterically abolished by the binding of β-galactosugars such as galactose and lactose to the lectin site of elastin binding protein [20,21]. To investigate the effect of lactose in fibroblast migration in response to bonito, bovine, and porcine elastin peptides, cells (1.0×10^{5} cells/ml) were exposed to 10 mM lactose at 37 °C for 2 h. An aliquot (50 μl) of cell suspension in DMEM was added to the upper compartment of the chamber, and then each of these elastin peptides was added to the lower compartment of the chamber. Migrated cells were counted in the same manner as described above after a 6-h incubation at 37 °C in a humidified atmosphere at 5% CO_2 in air.

Results

Elastin Peptides

Protein concentrations in NaCl and NaOH extracts obtained by the successive salt extractions with NaCl and alkaline extractions with NaOH, respectively, are shown in Figures 1 and 2. Five or six extractions with NaCl and NaOH were adequate for the removal of the salt-soluble proteins such as plasma proteins and for the removal of collagen, respectively, because the biuret reactions of the fifth or sixth NaCl and NaOH extracts were lower than 0.1 mg/ml protein. Molecular weights of elastin peptides were determined by gel filtration chromatography (Figure 3). As a result, the average molecular mass of elastin peptides obtained from bonito bulbus arteriosus was 200 kDa, and the average molecular masses of both elastin peptides obtained from bovine ligamentum nuchae and porcine aorta were 220 kDa. The amino acid compositions of elastin peptides obtained by partial hydrolysis of purified elastin peptides are summarized in Table 1. The contents of Gly, Pro, Ala, and Val residues; Asp and Glu residues; Lys, His, and Arg residues; and desmosine (Des) and isodesmosine (Ide) residues in bovine and porcine elastin peptides were in the range of 80–83%, 2.5–2.6%, 1.0–1.5%, and about 0.2% of total amino acid composition, respectively. Elastin peptides from bonito gave different contents of Gly, Pro, Ala, and Val residues; Asp and Glu residues; Lys, His, and Arg residues; and Des and Ide residues, that is, about 77, 3.4, 2.4, and 0.1%, respectively.

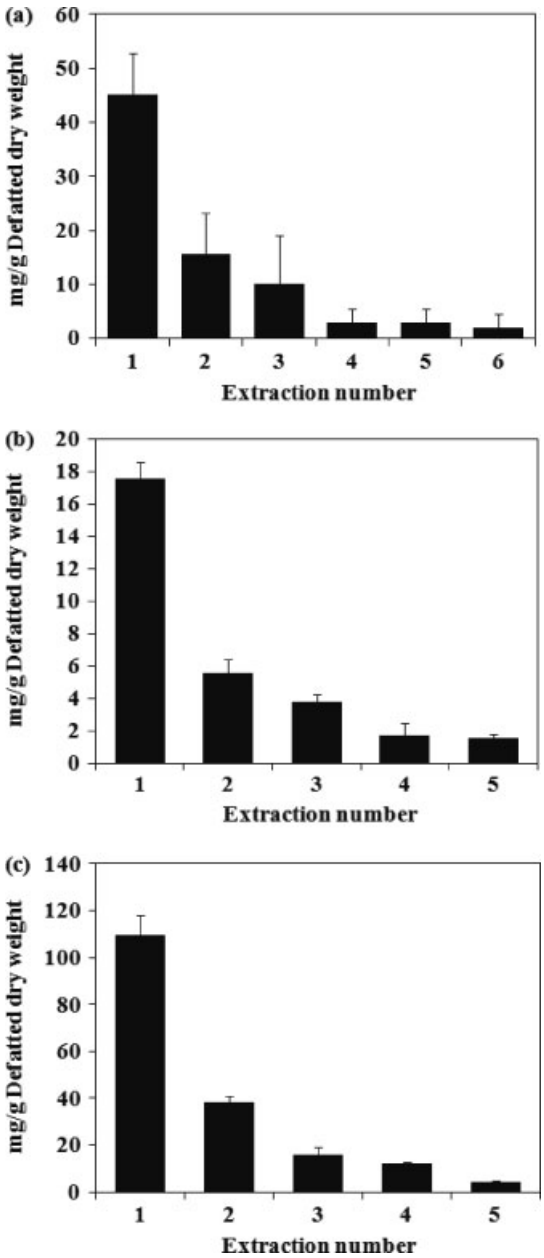

Figure 1. Protein content in salt-soluble extracts removed by NaCl-treatment of defatted dry tissues of bonito bulbus arteriosus (a), bovine ligamentum nuchae (b), and porcine aorta (c). All values are means ± SD obtained from triplicate experiments.

Inhibitory Action of Elastin Peptides on Platelet Aggregation

Inhibitory action of bonito, bovine, and porcine elastin peptides on platelet aggregation induced by collagen is given in Figure 4. Figure 4(a) exhibits inhibition profiles by elastin peptides at 20.9 mg/ml and Figure 4(b) shows the inhibitory activity of

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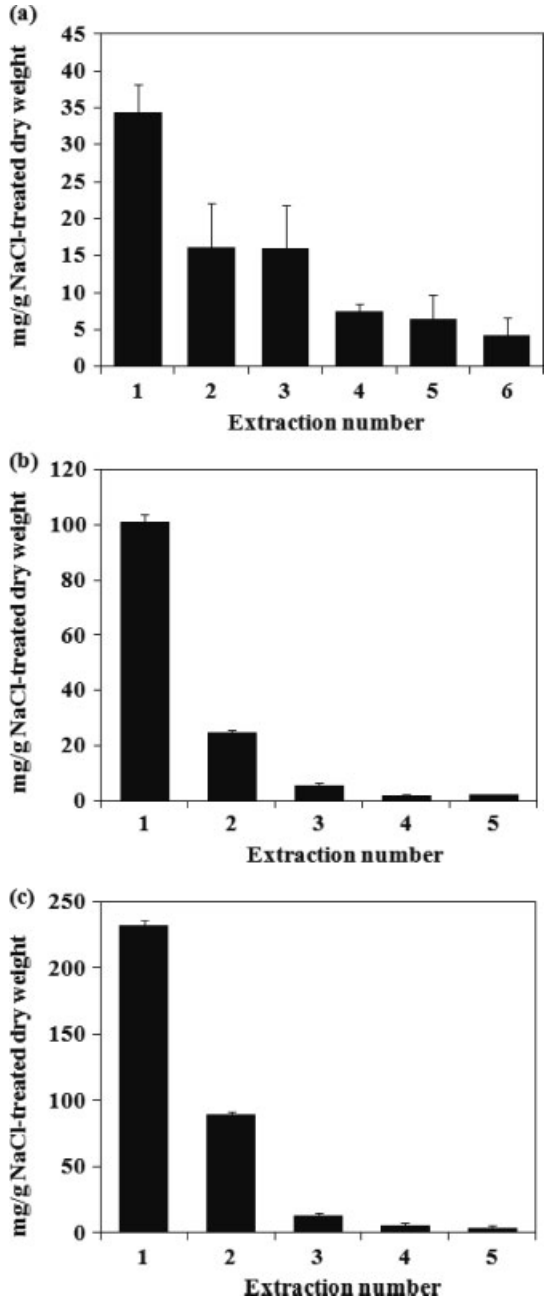

Figure 2. Protein content in alkaline-extracts removed by NaOH treatment of NaCl-treated dry tissues of bonito bulbus arteriosus (a), bovine ligamentum nuchae (b), and porcine aorta (c). All values are means ± SD obtained from triplicate experiments.

elastin peptides at various concentrations. All of bonito, bovine, and porcine elastin peptides inhibited collagen-induced platelet aggregation in a dose-dependent manner. The inhibitory activity of bonito elastin peptides at 20.9 mg/ml was about 1.9 times higher than that of porcine elastin peptides and about 2.1 times higher than that of bovine elastin peptides.

Response of Fibroblasts to Elastin Peptides

Effects of bonito, bovine, and porcine elastin peptides on human skin fibroblast proliferation are shown in Figure 5. All elastin peptides enhanced the proliferation of fibroblasts 3.5- to 4.5-

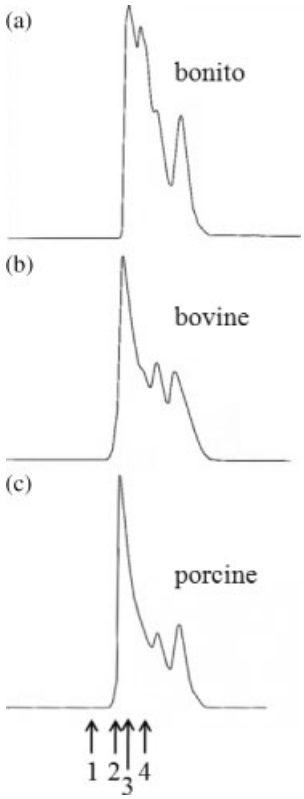

Figure 3. Chromatogram of elastin peptides by gel filtration chromatography. Chromatograms of (a) bonito elastin peptides, (b) bovine elastin peptides, and (c) porcine elastin peptides. Molecular mass standards were (1) thyroglobulin (669 kDa), (2) catalase (232 kDa), (3) L-lactate dehydrogenase (140 kDa), and (4) bovine serum albumin (66 kDa).

fold during treatment for 48 h at a concentration of 10 μg/ml, but to a lesser extent than TPA (10-fold). Since there were little differences in their effects on cell proliferation, an experiment on cell migration was performed using these elastin peptides.

The migration of human skin fibroblasts in response to bonito, bovine, and porcine elastin peptides in a concentration gradient ranging from 10^{-4} to 10^{4} μg/ml is shown in Figure 6. Bovine and porcine elastin peptides stimulated the migration of fibroblast, with the optimal response occurring at 10^{-1} μg/ml, while maximal response to bonito elastin peptides was observed at 10^{2} μg/ml, peaking at a higher concentration. The migratory activities of bonito elastin peptide at 10^{2} μg/ml and bovine and porcine elastin peptides at 10^{-1} μg/ml were 75% and 42–47% of fibronectin control, respectively.

As shown in Figure 7, pre-exposure of fibroblasts to 10 mM lactose, an antagonist of elastin receptor, depressed their ability to migrate in response to bonito, bovine, and porcine elastin peptides.

Discussion

Elastin in mature elastic fibers is an insoluble, cross-linked protein. In this investigation, the first step is the isolation of insoluble elastin from bonito bulbus arteriosus, porcine aorta, and bovine ligamentum nuchae. The salt-soluble proteins such as plasma proteins and the alkaline-soluble protein such as collagen in these tissues were removed by the successive salt extractions with NaCl and alkaline extractions with NaOH to yield a purified insoluble

Table 1. Amino acid compositions of elastin peptides from bonito bulbus arteriosus, porcine aorta, and bovine ligamentum nuchae[a]

Amino acids	Elastin peptides from bonito	Elastin peptides from bovine	Elastin peptides from porcine	Insoluble elastin from bovine [22]	Insoluble elastin from porcine [23]
Hyp	5	6	6	8	11
Asp	11	6	3	6	6
Thr	59	8	21	9	14
Ser	16	11	11	9	11
Glu	23	19	23	16	19
Pro	111	115	107	116	117
Gly	491	327	325	330	330
Ala	87	229	242	228	234
Val	77	159	128	132	120
Met	1	0	0	0	0
Ile	9	22	16	24	18
Leu	32	53	53	60	54
Tyr	31	6	21	6	16
Phe	22	29	29	30	33
Ide + Des[b]	1	2	2	15	3
His	1	0	0	1	1
Lys	4	4	8	3	6
Arg	19	6	7	6	6

[a] Values are expressed per 1000 amino acid residues.
[b] Ide, isodesmosine; Des, desmosine.

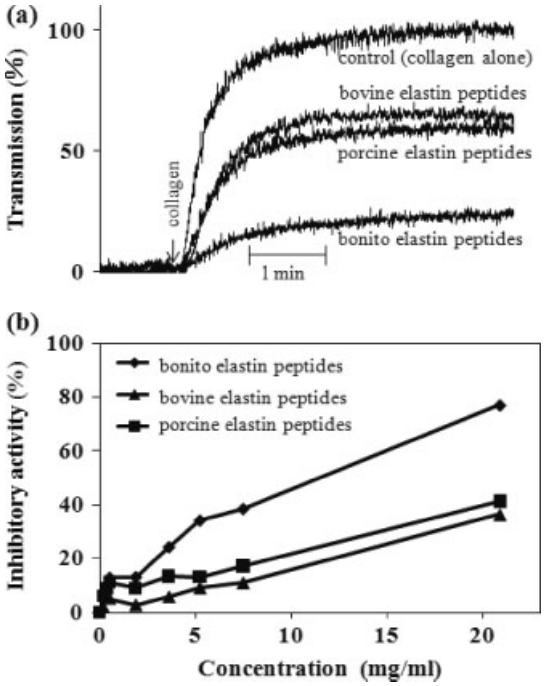

Figure 4. Inhibitory effects of bonito, porcine, and bovine elastin peptides on platelet aggregation induced by collagen. Each of elastin peptides was added to 0.25 ml of platelet-rich plasma (5×10^3 platelet/μl) before adding 7 μl of 0.5 mg/ml collagen. (a) Profiles of inhibition by elastin peptides at 20.9 mg/ml. (b) Inhibitory activity of elastin peptides at various concentrations. Results are expressed as percentages of control measurement using collagen alone.

elastin. In alkaline extractions from bonito bulbus arteriosus, milder conditions such as lower NaOH concentrations and lower temperatures were used in the NaOH treatment of NaCl-treated tissue to avoid the degradation of elastin. The second step is the preparation of elastin peptides from the purified insoluble elastin. The purified insoluble elastins from bonito bulbus arteriosus, porcine aorta, and bovine ligamentum nuchae were partially hydrolyzed by the successive treatment with oxalic acid according to the method of Partridge *et al*. [24]. The amino acid compositions of bovine and porcine elastin peptides obtained were in fair agreement with those of insoluble elastins reported previously [22,23]. Sage and Gray [25] have reported that elastins from mammalians such as pig, kangaroo, vole, dog, and dolphin have distinctive amino acid compositions, which are enriched in Gly (30–36%), Ala (23–25%), Val (10–13%), and Pro (9–12%), and which are deficient in hydrophilic amino acids and have higher crosslink contents (0.16–0.34%) and that elastins from teleosts such as black grouper, black cod, Ling cod, pacific yellowtail, starry flounder, carp, and king salmon are enriched in hydrophilic amino acids and Gly (40–45%), depleted in Ala (8–14%) and Val (5–8%) and have lower crosslink contents (0.05–0.13%). Their report suggested that amino acid composition of bonito elastin was similar to those of teleost elastins.

As to inhibition of collagen-induced platelet aggregation, bonito elastin peptides showed a higher inhibitory activity than bovine and porcine elastin peptides did. This result may be due to the distinct difference between the amino acid compositions of the bonito elastin peptides and the bovine and porcine elastin peptides. The higher content of hydrophilic amino acids such as Gly, Asp, Thr, Ser, and Arg and the lower content of hydrophobic amino acids such as Ala, Val, Ile, and Leu in bonito elastin peptides seem to result in the higher inhibition of platelet aggregation. As coacervation is the process of intermolecular hydrophobic order, the higher content of hydrophilic amino acid and the lower content of hydrophobic amino acids in bonito elastin peptides appear to suppress the coacervation process. Although the mode of inhibitory action of elastin peptides is not yet clear, Kimura *et al*. [26] suggested that the inhibition of thrombin-induced platelet aggregation by soluble elastin from bovine ligamentum nuchae might be due to the reduction of intraplatelet free calcium concentration [$(Ca^{2+})i$] through the inhibition of Ca^{2+} mobilization. Furthermore, Sekiya and Okuda [8] reported that the level of cAMP in platelets was not affected by soluble elastin, but we found that the cAMP level markedly increased when stimulated by collagen in the presence of elastin peptides (data not shown). cAMP is known as a second messenger inducing inhibition of platelet aggregation [27]. Recently, it was reported that splitomicin, which is derived from β-naphthol, inhibited thrombin or collagen-induced platelet aggregation by an increase in cAMP level and a decrease in intracellular Ca^{2+} mobilization [28]. It can, therefore, be presumed that the increase of cAMP level and the decrease of intracellular [$(Ca^{2+})i$] may play important roles in inhibition of platelet aggregation by bonito, bovine, and porcine elastin peptides.

Cell responsivenesses to bonito, bovine, and porcine elastin peptides were investigated. All elastin peptides enhanced the proliferation of human skin fibroblasts in a dose-dependent manner. Tajima *et al*. [15] reported that protein kinase C was involved in fibroblast proliferation in response to elastin-derived polyhexapeptide (VGVAPG)n. Blood and Zetler [29] suggested that the interaction of elastin peptides with tumor cells, which is mediated by a high-affinity cell receptor, is related to

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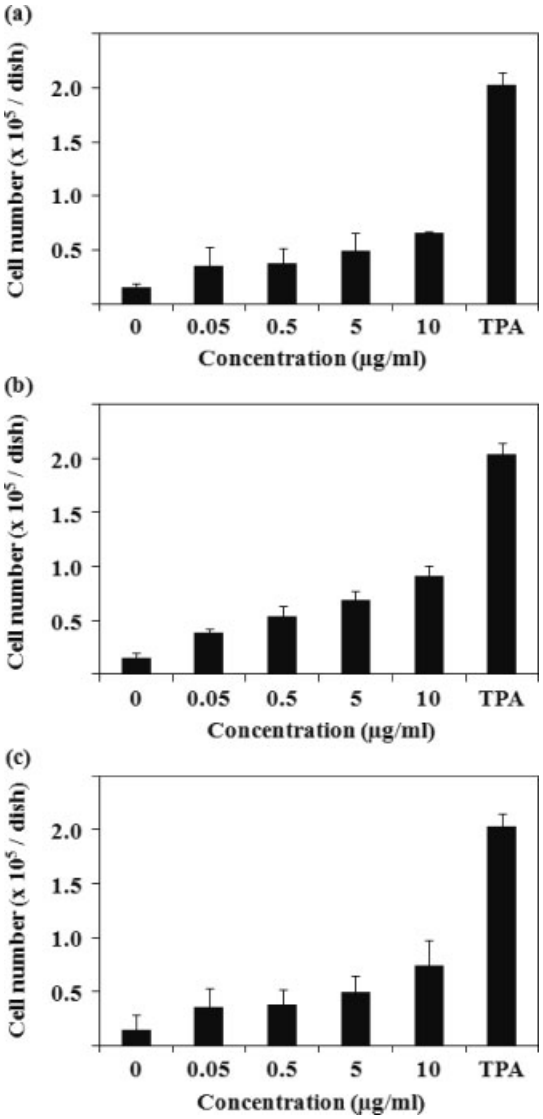

Figure 5. Effects of bonito, bovine, and porcine elastin peptides on fibroblast proliferation. Quiescent cultures in 0.5% FBS/DMEM were treated for 48 h with bonito elastin peptides (a), bovine elastin peptides (b), and porcine elastin peptides (c) at the concentrations indicated. TPA concentration was 10^{-6} μg/ml. All values are means ± SD obtained from triplicate experiments.

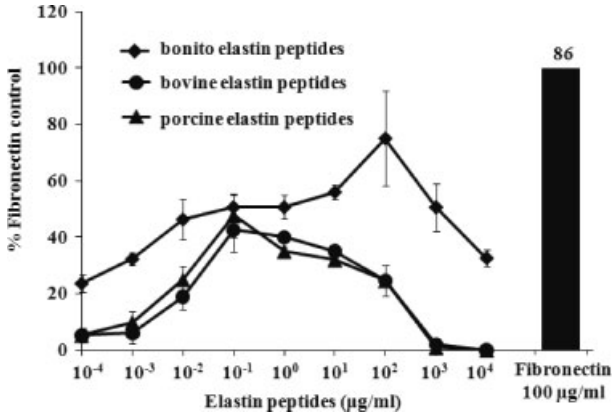

Figure 6. Fibroblast migration in response to bonito, porcine, and bovine elastin peptides. Net fibronectin migration was 86 cells per h.p.f. and background migration was 31 cells per h.p.f.

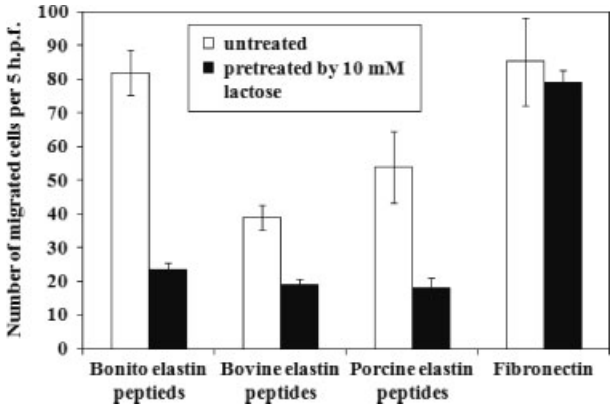

Figure 7. Inhibition by lactose of fibroblast migration in response to bonito, porcine, and bovine elastin peptides. The concentrations of bonito, porcine, and bovine elastin peptides were 10^{2}, 10^{-1}, and 10^{-1} μg/ml, respectively. The concentration of fibronectin was 100 μg/ml. The number of migrated cells was expressed as the net number of cells per 5 h.p.f. All values are means ± SD obtained from triplicate experiments.

membrane-bound protein kinase C. These evidences imply that all of bonito, bovine, and porcine elastin peptides enhance fibroblast proliferation via protein kinase C activation. All elastin peptides stimulated the migration of human skin fibroblasts, with biphasic response curves. The biphasic feature of the response curves may be attributed to several processes. One may be due to the saturation of cell-surface receptors that trigger the migrative response [30] and the other may be caused by the downregulation of the receptors [31].

Lactose-sensitive 67-kDa elastin binding protein has been known to be an elastin receptor [20]. Lactose is an antagonist of the elastin receptor and cause the dissociation of elastin binding protein from the cell membrane [19]. The pretreatment of fibroblasts by lactose suppressed the migration of fibroblasts to bonito, bovine, and porcine elastin peptides as shown in Figure 7. This finding suggests that the responsiveness of fibroblasts to all elastin peptides is mediated by 67-kDa elastin binding protein called elastin receptor. Further studies will be devoted to clarify the difference on the structures and functions between piscine elastin peptides from bonito bulbus arteriosus and mammalian elastin peptides from porcine aorta and bovine ligamentum nuchae. Furthermore, elastin peptides may become useful molecules, especially in biomaterials, aiming at replacing organs or tissues in which elasticity is crucial, e.g. blood vessels or skin.

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