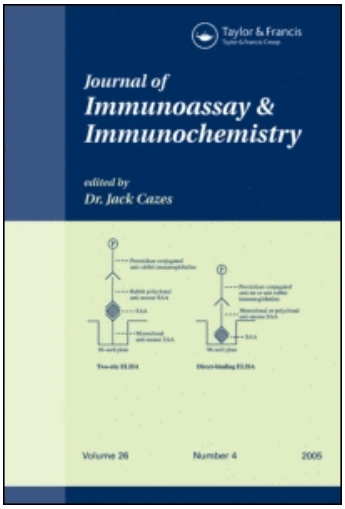

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Abstract: We have newly established 3 distinct murine monoclonal antibodies (MoAbs) against human soluble elastin by using chemically denatured immunogen isolated from human aorta; they are designated as HASG-2, HASG-30, and HASG-61-1. All of these MoAbs were highly reactive with soluble forms of native elastin in normal human serum. HASG-2 and HASG-61-1 MoAbs can recognize soluble bovine elastin as well as human antigen, but HASG-30 cannot. The sandwich enzyme-linked immunosorbent assay (ELISA) for human soluble elastin was developed with HASG-61-1 labeled with peroxidase and HASG-30 immobilized on the microplates. The circulating levels of soluble elastin in human healthy subjects (mean $\pm$ SD; 42.9 ± 19.9 ng/mL; $n = 85$) could be measured with full accuracy and reproducibility, and gradually increased with aging. The positive correlation between the levels and ages was statistically significant ($r = 0.581$, $p < 0.0001$). In addition, we could also determine the concentration of tropoelastin secreted from cultured human dermal fibroblasts accurately by this ELISA. This simple assay can be utilized for the routine clinical laboratory screening of patients with arteriosclerotic

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vascular diseases or to accurately determine the concentrations of tropoelastin secreted from cultured human cells.

Keywords: ELISA, Elastin, Tropoelastin, Dermal fibroblasts, Aging, Arteriosclerosis

INTRODUCTION

Elastin is the major extracellular component deposited in the arterial wall; it is synthesized and secreted by vascular smooth muscle cells.[1,2] Elastic fibers are assembled extracellularly with composition of elastin and microfibrillar proteins, including fibrillins and microfibril-associated glycoproteins.[2,3] Mature elastin itself is a polymer of enzymatically cross-linked monomers of tropoelastin, which is a soluble precursor protein with an approximate molecular weight of 70,000 and constitutes about 90% of the mass of elastic fibers.[2,3] Elastic fibers usually maintain elastic function for an entire lifetime, but occasionally, intravascular inflammation including macrophage accumulation may induce their proteolytic degradation leading to decrease elasticity of the aortic wall.[4,5] Previous reports have revealed that elastic fibers can be degraded by several proteases produced by vascular or inflammatory cells, and also that soluble elastin fragments generated from the damaged aortic tissues may be released into the circulation.[5–9]

Several attempts were made to determine soluble elastin fragments by using polyclonal animal antiserum raised against chemically solubilized elastin, in which the antibody specificity may not be uniform.[6–9] Most of the previously established anti-elastin MoAbs were utilized in the competitive immunoassays to measure the concentration of human serum antigens.[8–11] However, the circulating elastin levels in human subjects varied extremely among them, because most of these MoAbs may not recognize the antigenic epitopes commonly exposed on chemically solubilized and circulating form of native elastin.[12] There has been no sandwich-type immunoassays developed by using 2 distinct MoAbs for measuring the concentration of soluble elastin antigen in biological fluids. The aims of the study are (1) to create novel MoAbs to efficiently recognize soluble form of human native elastin in circulation, and (2) to establish a quick and reliable sandwich immunoassay system by using these MoAbs for determination of the concentration of soluble elastin antigens in biological fluids.

EXPERIMENTAL

Generation of MoAbs

Anti-human elastin MoAbs were newly created according to the standard hybridoma technology.[13] Commercially available human aortic soluble elastin (Elastin Product Company, Owensville, MO) prepared according to

the published method[14] was used as immunogen. The immunogen (100 μg) emulsified with aluminum adjuvant (Pierce Chemical, Rockford, IL) was injected into the BALB/c mice. The same injections were repeated twice for 4 weeks intervals. Three days after the last injection, splenocytes were surgically removed and then fused with SP-2/0 murine myeloma cells. High amounts of MoAbs were obtained from the serum-free hybridoma culture supernatants (Hybridoma-SFM medium; Gibco Life Technologies, Gaithersburg, MD). IgG purification was performed by using Protein-A-Sepharose column chromatography as previously described.[13]

We estimated the relative binding of each MoAb to human aorta-derived or bovine neck ligament-derived soluble elastin (Elastin Product Co.) immobilized on the 96-well microplates (Nunc, Roskilde, Denmark) by the standard indirect ELISA.[13] These antigens were solubilized in phosphate-buffered saline (PBS) at the final concentration of 10 μg/mL and added to the wells. After incubation at 4°C for 24 hours, all the wells were blocked with 1% skim milk/PBS. Each MoAb and control murine IgG was added to the well and kept at room temperature for 1 hour. The wells were washed 3 times with PBS, and then the bound antibodies were captured by anti-mouse IgG labeled with peroxides (Dako, Glostrup, Denmark). The relative bindings of MoAbs were estimated by measuring the color development of 3-, 3′-, 5-, 5′-tetramethyl-benzidine-2HCl (TMBZ) substrates (Sigma Chemical Co., St. Louis, MO), after their enzymatic reactions were stopped by adding 2N HCl solutions, in the T-max microplate reader (Molecular Devices Co., Sunnyvale, CA).

Blood Samples

Serum samples from healthy human subjects were obtained from ICI Japan Co., Ltd (Tokyo, Japan). Each blood sample was drawn after written informed consent was obtained. Serum was kept frozen at −80°C until used for assay. In addition, pooled citrated human plasma and normal human serum were purchased for the assay validation tests (Sigma). Five kinds of animal serum (bovine, goat, porcine, or rabbit) were commercially available (Cosmo Bio Co., Ltd, Tokyo, Japan). Serum of rhesus monkey was obtained in the primate section of our laboratory.

Sandwich ELISA

Two-site sandwich immunoassay was performed basically according to the previous method.[15,16] Briefly, one of the purified MoAbs was conjugated with EIA-grade horseradish peroxidase (Roche Diagnostics GmbH, Mannheim, Germany) as described elsewhere.[13] The other MoAbs were immobilized on the Maxsorp microplate (Nunc), and the wells were blocked with the reaction diluent (1% skim milk/PBS containing 2mM EDTA and 0.1% NaN_3).

Human aortic soluble elastin (Elastin Product Co.) solubilized in the reaction diluents was used as the assay standards. Blood specimens from healthy individuals or cell culture fluids were diluted 10 times by the reaction diluent, and then entered into the wells of the microplates. The standards (0, 0.69, 2.06, 6.17, 18.52, 55.56, 166.7, and 500.0 ng/mL) were also added to the other wells of the plates, simultaneously. After incubation at room temperature for 1 hour, the wells were washed 3 times by the Tris-buffered saline containing Tween-20 (TBS-Tween 20) and further incubated with peroxidase-labeled MoAbs at room temperature for 1 hour. Finally, the wells were washed 3 times by TBS-Tween 20 and then incubated with TMBZ substrate solution at room temperature for 10 minutes. The enzymatic reaction was stopped by adding 2N HCl solution to each well. The absorbance at 450 nm was measured at T-max microplate reader and the concentrations were calculated according to the calibration curve by the software (Soft T-max; Wako, Osaka, Japan).

Determination of Tropoelastin Secreted From Human Fibroblasts Cultured in vitro

Normal human dermal fibroblasts were obtained from Dainippon Pharmaceuticals (Osaka, Japan). The cells were routinely grown in RPMI-1640 medium (Sigma) containing 5% fetal calf serum (FCS) in the standard cell culture condition (5% CO_2). When cultures reached confluence, the cells were harvested by trypsinization and transferred into the 96-well microculture plates at a density of 1.0×10^4 cells/well. The cells were cultured for 48 hours initially, followed by the treatment of growth factors in the medium containing 0.5% FCS for 72 hours. Each of the commercially available growth factors, including recombinant human epidermal growth factor (EGF) purchased from Invitrogen (Carlsbad, CA), recombinant human interleukin-1β (IL-1β) from Strathmann Biotec AG (Germany), recombinant human insulin-like growth factor-1 (IGF-1) from R&D systems (Minneapolis, MN), and tumor growth factor-β (TGF-β) from Genzyme-Techne (Minneapolis, MN) was independently added to each well at the final concentration of 0.001, 0.01, 0.1, 1.0, or 10 ng/mL. The soluble elastin concentrations in these cell culture supernatants were measured by the ELISA as described in the above section. Cell proliferation rates were also estimated by the color intensity of water-soluble formasan WST-8 (Dojin Chemical Co., Kumamoto, Japan).

Statistical Analysis

The elastin concentrations are presented as mean $\pm$ SD. Kruskal-Wallis non-parametric test was performed for statistical multivariate comparisons

between the variables of the different groups. In this analysis, statistical significance was regarded as $p < 0.05$. We also utilized the ANCOVA test to analyze the effects of growth factors on cellular elastin synthesis with taking their cell proliferation effects into account. All calculations were performed with StatView 5.0 software (SAS Institute Inc., Cary, NC).

RESULTS

MoAbs

We finally established 3 hybridoma cell lines secreting the MoAbs (HASG-2, HASG-30, and HASG-61-1), highly reactive with human aortic soluble elastin utilized as immunogen. HASG-2 and HASG-61-1 were fully cross-reactive with bovine antigens, and otherwise HASG-30 was not (Fig.1). For quantitative assay for human soluble elastin, HASG-30 MoAbs as the solid-phase were combined with HASG-61-1 MoAbs as the detector (Fig. 2A), and this ELISA was designated as the 30-61 format. We found that HASG-2 can bind to the immunogen not competitively with HASG-61-1 (data not shown), and the sandwich ELISA could be performed using HASG-2 labeled with peroxidase and HASG-61-1 absorbed on the microplates (Fig. 2A), also designated as the 61-2 format. All of these MoAbs were satisfactorily reactive with human soluble elastin antigens in normal human circulation, because each of them in any of the two-site ELISA formats could efficiently bind to soluble elastin antigens present in human serum (Fig. 2). Soluble elastin antigens in any of the animal serum used here could not be captured in the 30-61 format

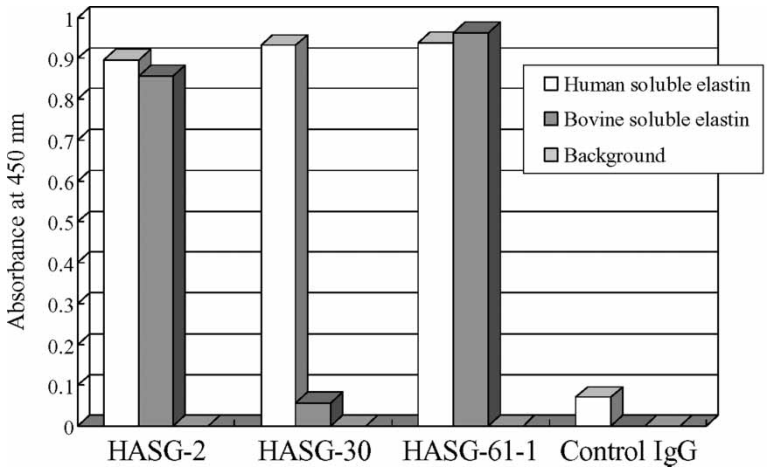

Figure 1. Relative reactivities of the established monoclonal antibodies to human or bovine soluble elastin immobilized on the microplates.

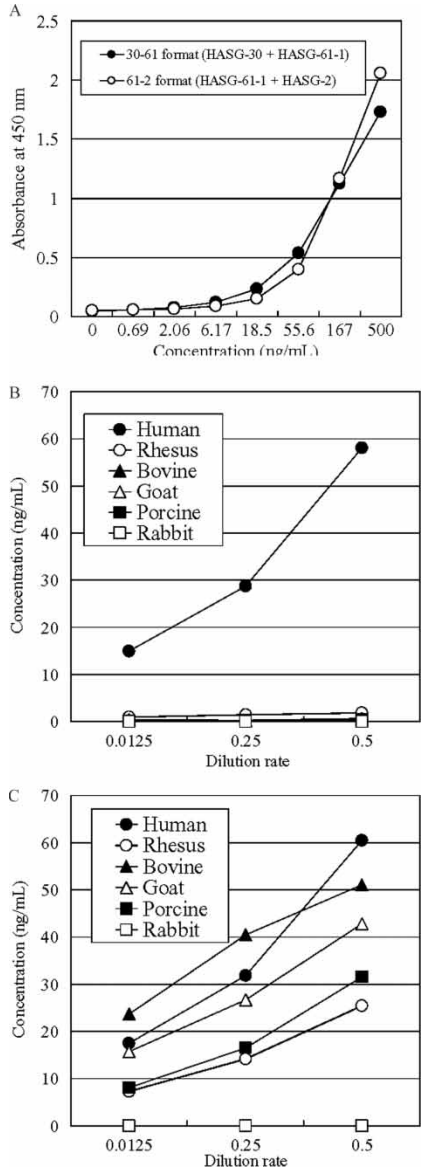

Figure 2. Typical standard curves of the ELISAs for human soluble elastin (A) and dilution curves of soluble elastin antigens in human and animal sera using the 30-61 format (B) or the 61-2 format (C). Representative standard curves were obtained in the 30-61 or the 61-2 ELISA formats using human aortic soluble elastin as an assay standard (A). Soluble elastin antigens in human serum could be detected similarly in the 30-61 (B) and the 61-2 ELISA format (C). Soluble elastin antigens in most of the animal sera were apparently detectable only by the 61-2 format, but those in rabbit sera was negligibly detected.

(Fig. 2B). On the other hand, soluble elastin in rhesus monkey, bovine, goat, or porcine serum were apparently detected by the 61-2 format (Fig. 2C), indicating that both of these two MoAbs can sufficiently cross-react with soluble elastin in these animal serum. We selected the 30-61 ELISA format in order to assay soluble elastin antigens in human biological fluids in the following experiments.

Precision of Assay and Recovery

We evaluated the precision of the 30-61 format by assaying 6 human serum samples 10 times each in a continuous series (intra-assay) or twice each in 10 consecutive assays (interassay). The intra-assay and interassay coefficients of variation ranged from 1.9% to 4.2% and 1.6% to 8.4%, respectively (Table 1). A series of plasma and serum samples containing high, medium, or low amounts of human aortic soluble elastin (Elastin Product Co.) were artificially constructed, and a pair of the serum or plasma samples were mixed at equal volumes (Table 2). Mean analytical recovery of 2 reconstructed serum samples was 98.0%, ranged from 89.3% to 102.5% (Table 2). The similar result was observed using citrated plasma specimens (103.0%, ranged from 90.7% to 123.4%). Another series of reconstructed plasma or serum samples was prepared to contain high, medium, or low amounts of human aortic soluble elastin, and they were serially diluted from 1/2 up to 1/128 in the reaction diluent, showed a linear recovery pattern in the assay for soluble elastin antigens (Fig. 3).

Table 1. Reproducibility of the 30-61 ELISA for human elastin

	Average (ng/mL)	S.D. (ng/mL)	C.V. (%)
A: Intra-assay variation (N = 10)			
Sample-1	275.8	11.58	4.2
Sample-2	194.5	5.23	2.7
Sample-3	91.5	2.79	3.1
Sample-4	23.5	0.45	1.9
Sample-5	5.1	0.15	3.0
Sample-6	1.6	0.05	2.9
B: Inter-assay variation (N = 10)			
Sample-1	278.7	11.95	4.3
Sample-2	191.1	5.95	3.1
Sample-3	89.2	1.47	1.6
Sample-4	23.1	1.82	7.9
Sample-5	5.2	0.43	8.4
Sample-6	1.55	0.06	4.0

Table 2. Recovery of measurement for elastin antigen in the 30-61 ELISA

	Concentration of soluble elastin (ng/mL)				% of recovery (A/B)
	Sample-1	Sample-2	Measured (A)	Expected (B)	
Plasma	187.0	110.2	144.7	148.6	97.4
	187.0	68.3	115.8	127.7	90.7
	110.2	68.3	94.7	89.3	106.0
Serum	173.3	102.3	141.2	137.8	102.5
	173.3	52.3	100.7	112.8	89.3
	102.3	52.3	75.2	77.3	97.3
Plasma	187.2	84.3	129.4	135.8	95.3
	84.3	2.8	53.8	43.6	123.4
	187.2	2.8	100.0	95.0	105.3
Serum	119.3	43.1	80.3	81.2	98.9
	119.3	8.4	63.7	63.9	99.7
	43.1	8.4	25.8	25.8	100.0

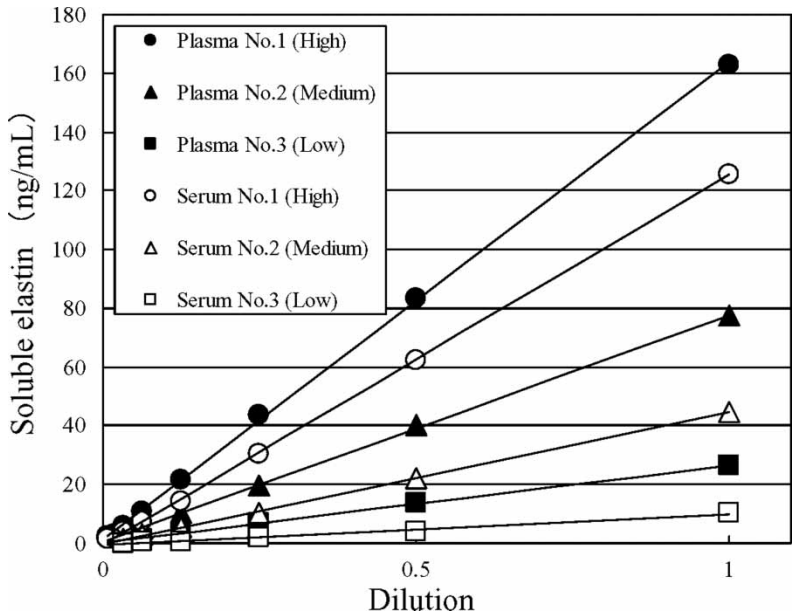

Figure 3. Dilution tests of reconstructed human serum or plasma samples containing different levels of soluble elastin. A series of pooled human serum or plasma were prepared to contain human soluble elastin antigens at final concentrations of high, medium or low. They were gradually diluted by the reaction diluents and their elastin concentrations were determined by the 30-61 ELISA format.

Measurement of Soluble Elastin Antigen in Healthy Subjects

Serum samples were obtained from 85 healthy individuals (mean age; 41.7 years, ranged from 22 to 66) and the circulating levels of soluble elastin were determined ($42.9 \pm 19.9\,ng/mL$). In 65 men (41.5 years, ranged from 23 to 66) and 20 women (42.3 years ranged from 22 to 60), the serum elastin levels gradually increased along with aging (Fig. 4). The positive correlation was statistically significant between the levels and ages in those subjects ($r = 0.581$, $p < 0.0001$). There was no significant difference between the levels in men and women (41.9 ± 20.5 and $46.2 \pm 17.8\,ng/mL$; $p = 0.1989$). We further analyzed the differences among 4 blood types, and no statistical difference was obtained among them (Kruskal-Wallis test).

Quantitative Analysis for Soluble Elastin in Cell Culture Fluids

The culture supernatants of human dermal fibroblasts were collected and the concentrations of soluble elastin antigen were measured by the 30-61 format (Fig. 5). After the removal of supernatants, their relative cell proliferations were estimated by the color developments of WST-8 reagents (Fig. 6). Cellular secretion of soluble elastin was dose-dependently up-regulated by TGF-β (0.001 to 1.0 ng/mL; Fig. 5A). Our statistical analysis (ANCOVA) demonstrated, however, TGF-β supplementation induced no significant increase of tropoelastin secretion from fibroblasts, considering its effect on

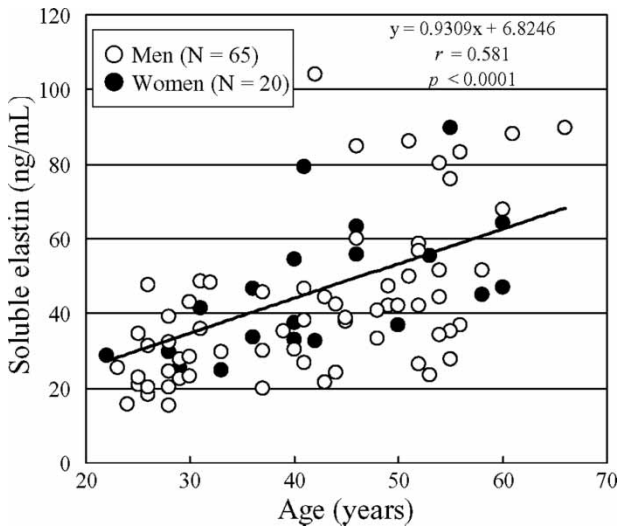

Figure 4. Relationship between the soluble elastin levels and ages in the 85 healthy subjects. The correlation was regarded as significant by the linear regression analysis.

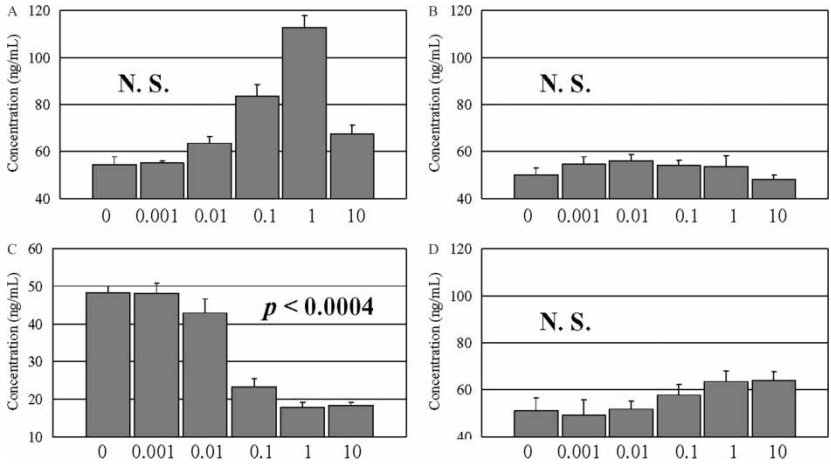

Figure 5. Effects of growth factors, such as TGF-β (A), IGF-1 (B), EGF (C), or IL-1β (D), on soluble elastin secretion from in vitro cultured human dermal fibroblasts. The vertical boxes and bars in the graphs indicated the mean concentrations and SDs of soluble elastin antigens in five independent experiments, respectively. The final concentration of each growth factor was indicated below the graphs at degree of nanogram per milliliters (ng/mL). The vertical scale of the graph C ranged between 10 and 60 ng/mL, and those of the other graphs did between 40 and 120 ng/mL. The concentrations of soluble elastin in the EGF treatment ($\geq$0.1 ng/mL) significantly decreased as compared to those in untreated control cells (ANCOVA analysis; $p < 0.0004$).

cell growth. On the contrary, the treatment by EGF ($\geq$0.1 ng/mL) significantly enhanced cell growth (Fig. 6C) while reduced the concentration of soluble elastin drastically (Fig. 5C). Neither IGF-1 nor IL-1β affected the cellular elastin secretion (Fig. 5B and 5D), taking its cell proliferation effects into account (Fig. 6B and 6D). Dose-dependent cell proliferation was statistically significant (Kruskal-Wallis non-parametric test) in the cells treated with TGF-β (Fig. 6A, $p < 0.008$), IGF-1 (Fig. 6B, $p < 0.005$), EGF (Fig. 6C, $p < 0.0001$), or IL-1β (Fig. 6D, $p < 0.0001$). Statistical ANCOVA analysis revealed that the cellular secretion of soluble elastin was significantly reduced only by EGF supplementation ($\geq$0.1 ng/mL; $p < 0.0004$).

DISCUSSION

We established 2 novel sandwich ELISA systems: namely, the 30-61 and the 61-2 format. The standard curve obtained in the 30-61 ELISA format was ideal for analysis of human soluble elastin antigens, as the HASG-30 MoAb can only recognize human antigen specifically (Figs. 1, 2A, and 2B). In this format, the intra-assay reproducibility within the range of standards was

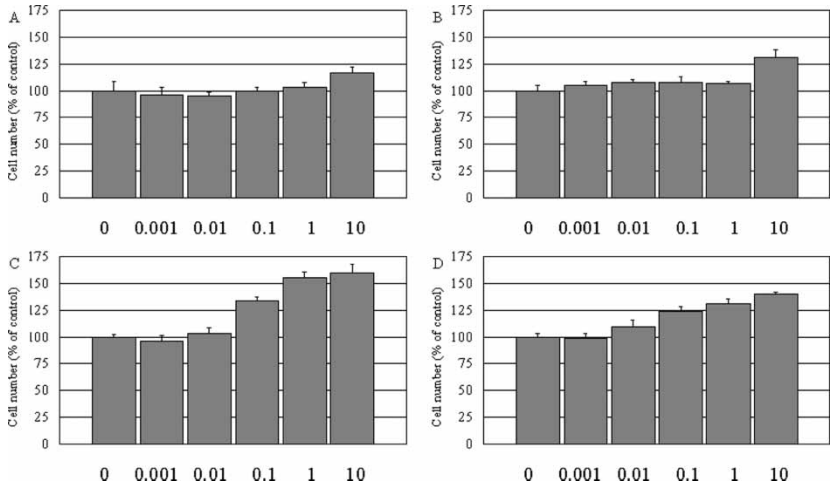

Figure 6. Effects of growth factors, such as TGF-β (A), IGF-1 (B), EGF (C), or IL-1β (D), on cell proliferation of in vitro cultured human dermal fibroblasts. The vertical boxes and bars in the graphs indicated the percents and SD of control cell growth in five independent experiments, respectively. The final concentration of each growth factor was indicated below the graphs at degree of nanogram per milliliters (ng/mL). Dose-dependent cell proliferation was found to be statistically signioficant (Kruskal-Wallis non-parametric test) in the cells supplemented with TGF-β (A: $p < 0.008$), IGF-1 (B: $p < 0.005$), EGF (C: $p < 0.0001$), or IL-1β (D: $p < 0.0001$).

regarded to be sufficient for the routine clinical analysis (Table 1). In contrast, the 61-2 format was apparently suitable for detection of soluble elastin antigens in animal models including bovine, goat, porcine, or rhesus monkeys (Fig. 2C). Unfortunately, the soluble form of elastin in rabbit serum could not be detected by this format. There has been no validated report to measure soluble elastin levels in animal biological fluids, and thus the 61-2 ELISA format can be potentially utilized in vascular biological research using some animal models in future.

The levels of circulating soluble elastin in healthy human subjects gradually increased with aging, and the correlation between them was regarded as statistically significant ($p < 0.0001$; Fig. 4). The circulating elastin concentration has been suggested as an indicator for aging,[17] whereas some controversial result was also reported.[7] The present result supports that the circulating soluble elastin level can be available as aging indicator, possibly for monitoring vascular aging.

Furthermore, soluble elastin in circulation have been evaluated to be a clinical marker of several diseases, including obstructive lung disease and arteriosclerosis.[7,17] It was also reported that this marker should be utilized for diagnosis of aneurysms.[8] Our recent study has already revealed that the soluble elastin levels in circulation were significantly elevated in the

patients with acute aortic dissection.[18] The relationship between vascular aging and arteriosclerotic events was so complicated as to be resolved for elder healthcare,[19,20] and the soluble elastin levels in circulating blood may inform us of some clinical condition of vascular walls.

The soluble form of elastin released from human dermal fibroblasts cultured in vitro can be recognized as a tropoelastin, the precursor of elastin. It can be readily and accurately detected in the 30-61 format (Fig. 5). Although FCS was supplemented into the culture medium, residual bovine soluble elastin in the medium could not interfere human tropoelastin antigens to bind to immobilized HASG-30 MoAbs in the ELISA (Figs. 1 and 2B). The effects of 4 kinds of growth factors on cellular tropoelastin production were also assessed in the standard cell culture condition supplemented with FCS (Figs. 5 and 6). The augmented or inhibitory effect of TGF-β (0.01 to 1.0 ng/mL) or EGF ($\geq$0.1 ng/mL), respectively, was consistent with the previous reports.[21–24] We have found here for the first time that maximum amounts of TGF-β can apparently induce negative effect of cellular tropoelastin production (Fig. 5A), and this mechanism should be resolved in some further studies in order to realize the physiological action of TGF-β.

There has been no universal standardized method to measure the amounts of soluble elastin antigens in human circulating bloods, because the previous measurements of elastin-derived peptides in human body fluids have differed by a factor of 1000.[25] As we presented the assay validation data (Fig. 3, Tables 1 and 2), the structure of circulating elastin antigen generated in vivo can be fully recognized by our newly established MoAbs in the 30-61 ELISA. In addition, the concentrations of tropoelastin, non-degraded soluble form of elastin, possibly secreted from human dermal fibroblasts could be apparently determined by this ELISA format, suggesting native protein structure of tropoelastin can also be recognized by the 2 MoAbs, HASG-30 and HASG-61-1. Our present method may clarify some characters or natures of circulating elastin antigen in the future biochemical research.

In conclusion, the newly developed MoAbs-based ELISA systems are useful to determine the concentration of soluble elastin antigens in several biological fluids with full reliability and reproducibility. The immunodetectable soluble elastin in circulation may be originated in tropoelastin released from varied cell types, which have been recently demonstrated to generate elastogenesis in human arteriosclerotic vascular diseases.[26]

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