Stimulation of cell proliferation and autoregulation of elastin expression by elastin peptide VPGVG in cultured chick vascular smooth muscle cells

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Abstract Synthetic elastin peptides, VPGVG or its polymer $(VPGVG)_n$, enhanced the proliferation of smooth muscle cells 1.5-fold during 48 h treatment at the concetrations over 10^{-6} M or 1.0 μg/ml, respectively. Monomeric and polymeric VPGVG sequences reduced elastin synthesis and its mRNA level to one-third and one-half of control respectively under the conditions in which the proliferation of cells were enhanced, but did not change collagen synthesis as measured by bacterial collagenase digestion. The elastin-specific autoregulation by elastin fragments may reflect the feedback regulation of elastin expression which may play an essential role in elastin metabolism under the normal and diseased conditions.

Key words: Elastin fragment; Elastin synthesis; Smooth muscle cell

1. Introduction

Elastin is a major connective tissue component in aortic wall and confers elasticity to the tissues [1]. Overproduction of elastin is thought to be responsible for the development of atherosclerosis [2]. The synthesis of elastin has been demonstrated to be regulated by many growth factors or cytokines [3]. However, understanding of overall regulation of elastin synthesis is not sufficient.

Elastin has unique repeating sequence in the hydrophobic region; tetrapeptide VPGX (X = G or A), pentapeptide VPGVG, hexapeptide XPGVGV (X = A or V), and nonapeptide VPGXGVGAG (X = L or F). Pentapeptide VPGVG is the only repeating sequence present in the elastin molecules of all animal species analyzed including human, bovine, porcine and chicken [4–9]. VGVAPG is a hexapeptide repeated multiple times in the human, bovine and porcine elastin molecules but not present in chicken elastin molecule [4,10]. This sequence is active as a chemoattractant for monocytes, elastin-producing fibroblasts [11] and tumor cells [12], and modulates protein kinase C activity in lung carcinoma cells [13]. However, it is not known whether or not the elastin fragments act on the biosynthetic properties of elastin of the cells.

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Abbreviations: BAPN, β-aminopropionitril; EDTA, ethylenediaminetetraacetic acid; NEM, *N*-ethylmaleimide; NMR, nuclear magnetic resonance; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; SSC, 0.15 M sodium chloride and 0.015 M sodium citrate; TCA, trichloroacetic acid; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

In this studies, we synthesized a pentapeptide VPGVG which is present in chicken elastin molecule and repeats multiple times, and found that the elastin fragment could modulate the biosynthetic phenotype of elastin at a pretranslational level and stimulate cell motility in the monolayered chick vascular SMCs. This is the first report concerning the autoregulation of elastin expression by an elastin fragment.

2. Materials and methods

2.1. Cell culture and treatment with the peptides

SMCs were isolated from aortas of 20-day-old chick embryos by a serial enzyme digestion (0.05% bacterial collagenase and 0.025% pancreatic elastase) [14]. The cells were plated at a density of 2.2×10^5 cell/cm^2 in 35- or 100-mm diameter dish (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. The cells at a primary culture were used in this experiments, unless indicated otherwise. To bring the cells to quiescent state, cultures of confluent cells were placed in 0.5% FBS for 48 h, then treated with various concentrations of elastin-derived peptides or TPA for indicated periods.

2.2. Effects of elastin peptides on cell proliferation

Cells were treated with trypsin (0.25%) and the cell number was determined with a Coulter counter.

2.3. Synthesis of elastin peptides

The peptides, $(GVP)_2$, VPGVG, and VGVAPG were synthesized by a solid-phase method. $(VPGVG)_n$ and $(VGVAPG)_n$ were synthesized as previously described [15,16]. The average value of n in both polymers was determined to be greater than 40 by NMR analysis.

2.4. Synthesis of elastin

The cultures treated with various peptides were labeled with [3,4-^{3}H]valine (20 μCi/ml) (2.1 TBq/mmol; Amersham) for the last 6 h of the treatment in valine-free DMEM (Gibco).

The proteins in the medium and cell extracts with 0.5 M acetic acid were separately precipitated with 30% ammonium sulfate in the presence of protease inhibitor cocktails (1 mM EDTA, NEM and PMSF), and resolved on 4–15% gradient SDS-PAGE followed by fluorography. Elastin synthesis relative to total protein synthesis was determined by scanning the fluorograms with a densitometer (Cliniscan, Helena Lab.) [17]. The cell number was determined after trypsin treatment with a Coulter counter. The amount of sample applied to SDS-PAGE was normalized to the cell number.

2.5. Synthesis of collagen

Cells were labeled with [2,3-^{3}H]proline (50 μCi/ml) (4.0 TBq/mmol; Amersham) for the last 6 h of the treatment in the presence of ascorbic acid (50 μg/ml). The proteins from medium and cell layer were combined and stored −20°C until ready for analysis. The amount of radioactivity incorporated into collagen was determined using purified bacterial collagenase [18] as previously described [19].

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2.6. Northern blot analysis

Total RNA was extracted from cultured cells as described previously [20] and stored at the concentration of 1 μg/μl at −80°C. RNA was denatured in 1 M glyoxal at 50°C for 1 h and resolved by electrophoresis on 1% agarose gel (10 μg RNA/lane), then blotted to nylon membrane filters (Pall Biosupport). The filters were hybridized in the solution (50% deionized formamide, 5 × Denhardt's solution, 0.1% SDS, 5 × SSC and 100 μg/ml tRNA) at 42°C for 24 h with appropriate ^{32}P-labeled cDNA probes. The cDNAs for chicken elastin [21], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [22] were labeled with [^{32}P]dCTP to a specific activity of ~ 10^8 cpm/μg DNA using multiprime DNA labeling system (Amersham). Filters were washed with two changes of 2 × SSC/0.1% SDS at room temperature for 30 min, then with two changes of 0.1 × SSC/0.1% for 30 min. Filters were air-dried and autoradiographed. The autoradiograms were scanned with a densitometer.

3. Results

The elastin peptides, VPGVG monomer and polymer (Fig. 1c and e) enhanced SMC proliferation (1.5-fold) to the same extent as TPA (1.6-fold) (Fig. 1a). The peptides, monomer and polymer of VGVAPG, showed no significant effect on cell proliferation (Fig. 1d and f). Treatment of the cells with $(VGV)_2$ resulted in a slight increase in cell proliferation but to lesser extent than VPGVG monomer or polymer (Fig. 1b). Based on these results, further experiments on elastin expression were performed with monomer and polymer of VPGVG, using VGVAPG as a negative control.

Elastin level in the culture medium was inhibited by the treatment with VPGVG dose-dependently (Fig. 2a). Elastin level in the cell layer was slightly reduced (Fig. 2b). Relative elastin synthesis in the combination of the medium and cell layer measured by autoradiograms demonstrated that maximum inhibition of one third of control was achieved at the concentration of 10^{-6} M during 48 h treatment (Fig. 2, right panel). In contrast, VGVAPG exhibited no significant effect on

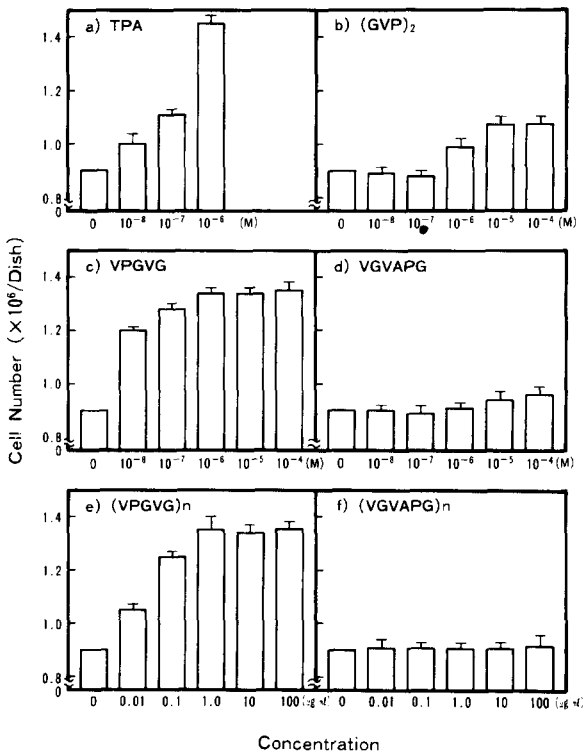

Fig. 1. Effect of TPA and various elastin peptides on cell proliferation. Quiescent cultures were treated for 48 h with TPA (a) or various elastin peptide (b–f) at the concentrations indicated. At the termination of incubation, cells were trypsinized and the numbers of cells were counted. Values are mean ± deviations obtained from triplicate experiments.

elastin synthesis (Fig. 3a and b). Similar results were obtained with the polymers of VPGVG and VGVAPG (Fig. 4a and b).

Neither VPGVG nor VGVAPG resulted in a significant

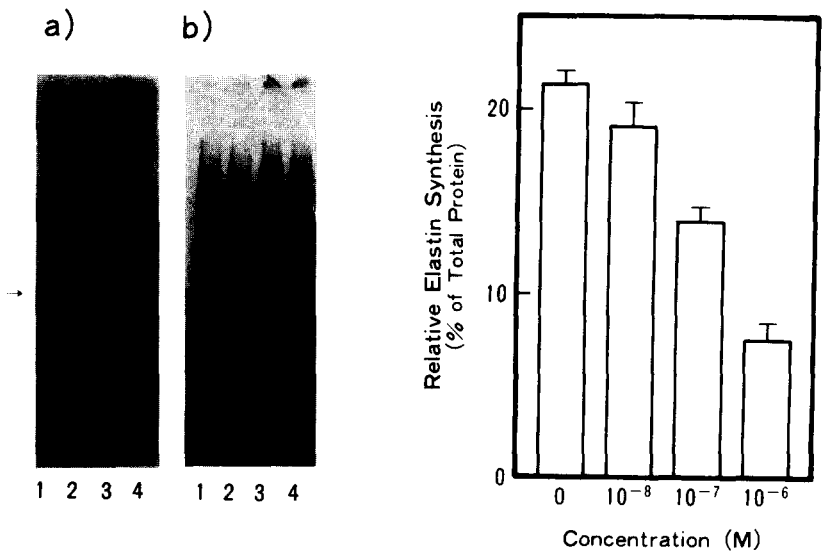

Fig. 2. Effect of elastin peptide, VPGVG, on elastin synthesis. Cultures were treated for 48 h with elastin fragment VPGVG at the concentrations of 0 (lane 1), 10^{-8} (lane 2), 10^{-7} (lane 3) and 10^{-6} M (lane 4), then labeled with [^{3}H]valine for the last 6 h. The proteins in the medium (a) and cell layer (b) were processed to SDS-PAGE followed by autoradiography. Arrows indicate the position of elastin. Autoradiograms were scanned with a densitometer. Relative elastin synthesis in the medium and cell layer was combined and expressed as a percentage of total protein synthesis (right panel). Values are mean ± deviation from duplicate experiments.

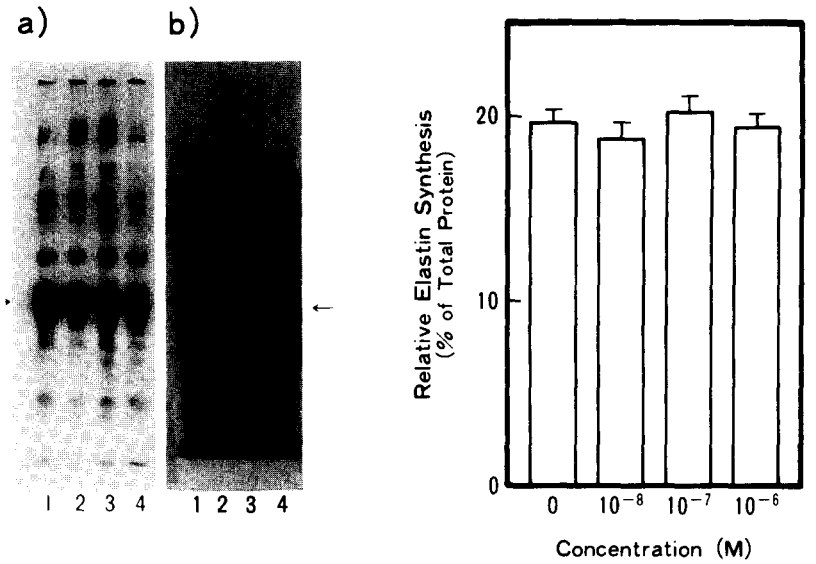

Fig. 3. Effect of elastin peptide, VGVAPG, on elastin synthesis. Cultures were treated for 48 h with elastin peptide VGVAPG at the concentrations of 0 (lane 1), 10^{-8} (lane 2), 10^{-7} (lane 3) and 10^{-6} M (lane 4), then labeled with [^{3}H]valine for the last 6 h. The proteins in the medium (a) and cell layer (b) were processed to SDS-PAGE and relative elastin synthesis was determined as described in Fig. 2 (right panel). Arrows indicate the position of elastin.

change in collagen and total protein syntheses as measured by bacterial collagenase digestion (Table 1).

The level of elastin mRNA was also reduced with the treatments of both form of VPGVG by one half of control at the concentration of 10^{-6} M for 48 h treatment (Fig. 5a and b, upper panels). In contrast VGVAPG did not alter elastin mRNA level at the concentrations between 10^{-8} and 10^{-6} M (Fig. 5c and d, upper panels). Both peptides did not change the level of GAPDH mRNA (Fig. 5a–d, lower panels).

4. Discussion

It has been reported that tropoelastin and elastin digests produced by elastase are chemoattractants for monocytes and fibroblasts [23] or elastin degradation products (K elastin) activate calcium influx in monocytes, fibroblasts and SMCs [24,25]. Although there is no direct evidence that elastin fragments used in this experiments exist as degradation products in vivo, these previous reports suggest that elastin degradation products actually exert an effect on cell migration.

The results obtained here clearly demonstrated that exogenously added elastin fragment. VPGVG and its polymer, stimulated cell proliferation and, at the same time, inhibited elastin expression. The simultaneous modulations for cell proliferation and elastin expression have been demonstrated; potent stimulators for SMC proliferation like EGF [26], angiotensin II [27], high K^+ concentration [17] and TPA (Wachi, H. and Tajima, S., in preparation) have been reported to inhibit elastin synthesis by SMCs. Therefore the decreased elastin expression by elastin fragment may be related to its stimulatory effect on cell proliferation.

The inhibition of elastin expression by elastin fragments may

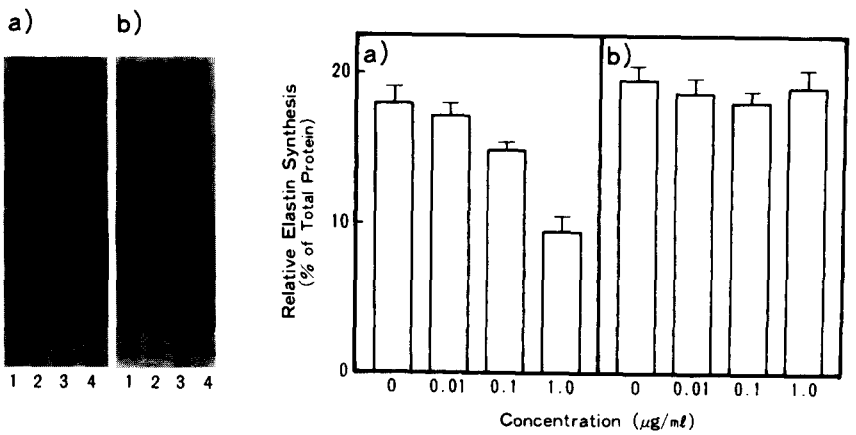

Fig. 4. Effect of elastin peptide polymers on elastin synthesis. Cultures were treated for 48 h with $(VPGCG)_n$ (a) or $(VGVAPG)_n$ (b) at the concentrations of 0 (lane 1), 0.01 (lane 2), 0.1 (lane 3) and 1.0 μg/ml (lane 4), then labeled with [^{3}H]valine for the last 6 h. The proteins in the medium and cell layer were processed to SDS-PAGE and relative elastin synthesis was determined as described in Fig. 2 (right panel). Autoradiograms of medium alone were shown in this figure.

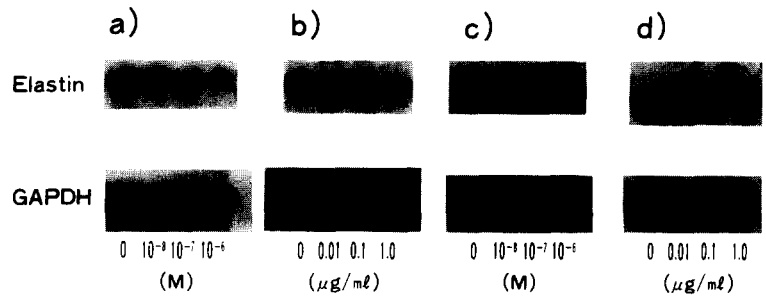

Fig. 5. Elastin mRNA level of the cells treated with the elastin fragment. Cells were treated with VPGVG (a), $(VPGVG)_n$ (b), VGVAPG (c) or $(VGVAPG)_n$ (d) for 48 h at the concentrations indicated. RNA was isolated and resolved on 1% agarose gel electrophoresis, then blotted onto nitrocellulose filters. The membrane was hybridized with ^{32}P-labeled elastin (upper panels) and GAPDH (lower panels) cDNAs. The blots were autoradiographed and scanned with a densitometer.

reflect the negative feedback regulatory mechanism by which elastin synthesis is controlled under normal and the diseased states. The modulation may be important to maintain a fine balance between synthesis and degradation of elastin in the normal condition and, moreover, to balance the accumulated elastin in the elastogenic tissues such as atherosclerosis. Negative autoregulation of elastin gene expression has been indirectly suggested when elastin secretion was inhibited by monensin [28] or elastin cross-linking formation was inhibited by the addition of BAPN [29]. Our results provided a direct evidence that the treatment of SMC with elastin fragment VPGVG resulted in the autoregulation of elastin expression. Similar autoregulations have been also reported in collagen synthesis in which *N*-propeptide of type I procollagen inhibited collagen synthesis and mRNA activities [30–32].

The modulations were found in the sequence VPGVG but not in VGVAPG. Since chicken tropoelastin has no repeating hexa- or nonapeptide [4], VPGVG appears to be an essential sequence for autoregulation of elastin synthesis and may be specifically recognized by chick SMCs. The modulation may be specific to elastin molecule, since collagen and total protein syntheses were essentially unchanged.

Although we have not studied the reactivity of elastin fragment VPGVG with SMC cellular membrane, the peptide is highly likely to interact with cell membrane because VGVAPG, a hexapeptide repeated multiple times in bovine, porcine and human elastin molecules has been already shown to bind to elastin receptors in the cell membrane [12]. VPGVG sequence is the ubiquitous pentapeptide found in the elastin molecules of all animal species analyzed. Unlike other repeating elastin fragments, it has a unique elastic property in vitro [33,34], suggesting that VPGVG plays an essential role in elastin metabolism in normal and diseased states as well as in maintaining the elastic structure.

Table 1
Effect of elastin peptides on collagen synthesis

Peptides	Concentration (M)	Collagen (cpm/cell)	Total protein (cpm/cell)	Relative collagen synthesis (%)
None		0.62 ± 0.03	1.4 ± 0.2	44
VPGVG	10^{-7}	0.60 ± 0.05	1.4 ± 0.2	43
	10^{-6}	0.60 ± 0.03	1.4 ± 0.3	43
VGVAPG	10^{-7}	0.68 ± 0.04	1.5 ± 0.3	45
	10^{-6}	0.60 ± 0.05	1.4 ± 0.4	43

Cultures were treated for 48 h with polymeric elastin peptides at the concentrations of 0.1 or 1.0 μg/ml, then labeled with [^{3}H]proline for the last 6 h in the presence of ascorbic acid (50 μg/ml). The proteins in the medium and cell layer were combined and collagen synthesis was determined by bacterial collagenase digestion. Relative collagen synthesis was expressed by collagen synthesis/total protein synthesis. Values are mean ± deviation from duplicate experiments.

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