LECTURE

Modulation of Elastin Expression and Cell Proliferation in Vascular Smooth Muscle Cells in vitro

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Abstract. Elastin expression in cultured vascular smooth muscle cell (VSMC) was found to be enhanced by potent inhibitors of VSMC proliferation including minoxidil, heparin and retinoic acid. By contrast, elastin expression was declined by potent stimulators of VSMC proliferation like epidermal growth factor, high K⁺, angiotensin II and phorbol ester. To elucidate the relationship between elastin expression and cell proliferation, the elastin expression in the different cell growth states brought by cell-synchronizing culture or suspension culture, a culture system independent of potent modulators of VSMC proliferation. Elastin was found to be expressed maximally at G0 and minimally at G2/M phases, suggesting that cell growth state regulates elastin expression in VSMC culture. Synthetic elastin peptide VPGVG or its polymeric form (VPGVG)n enhanced VSMC proliferation, which resulted in the reduction of elastin expression. The results suggests that elastin fragment regulates VSMC proliferation. These correlation between elastin expression and cell growth state may play an essential role in elastin metabolism under the normal and diseased conditions. (Keio J Med 45 (1): 58-62, March 1996)

Key words: elastin, smooth muscle cell, cell proliferation, elastin peptide

Introduction

The proliferation of vascular smooth-muscle cells (VSMCs) is a frequent consequence following endothelial injury, and is thought to be an important early pathogenic event in the evolution of the atherosclerotic plaque. VSMC proliferation in normal circumstances has been reported to be regulated by many mitogens, including epidermal growth factor (EGF),⁵ angiotensin II,⁶ platelet-derived growth factor⁷ and transforming growth factor β (TGFβ).⁸ Elastin is a major constituent of extracellular matrix in aortic wall and plays a crucial role in tissue elasticity. Overproduction of elastin is thought to be responsible for the development of atherosclerosis.⁴ The synthesis of elastin has been demonstrated to be regulated by many growth factors or cytokines; TGFβ,⁵ insulin-like growth factor,⁹ EGF⁴ and interleukin 1.¹⁰ These potent regulators for elastin synthesis play a key role in elastogenesis during atherosclerosis.

In this study, we attempted to clarify the relationship between cell proliferation and elastin expression and examined whether cell proliferation can influence elastin expression or elastin expression can influence cell proliferation.

Elastin Expression Is Modulated by the Modulators of VSMC Proliferation

VSMCs were isolated from aortas of the 20-day-old chick embryos by a serial enzyme digestion.¹¹ The cells were plated at a density of 2.2 × 10⁵ cell/cm² in 35 diameter tissue culture dishes and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells of primary culture were used in this experiment unless indicated otherwise. To bring the cells to quiescence, cultures of confluent cells were placed under a serum starvation condition (0.5% FBS) for 48 h (quiescent cells). To bring the cells to proliferative, quiescent cultures were subsequently placed in 10% FBS medium for 24 h (proliferating cells). Quiescent cultures were treated for 48 h with potent stimulators of VSMC proliferation including EGF, high potassium salt, angiotensin II (Ang II) or TPA. Proliferating cultures were treated for 24 h with potent inhibitors of VSMC proliferation like minoxidil, heparin
or retinoic acid.

The cells at a quiescent or proliferating state were labeled with [3,4-3H]valine (20μCi/ml) for 6h in valine-free DMEM supplemented with 0.5% FBS for quiescent cells or 10% FBS for proliferating cells. The proteins in the medium and cell layer was precipitated with ammonium sulfate (176mg/ml) in the presence of protease inhibitor cocktail containing 1mM of EDTA, NEM and PMSF. Proteins in the medium and cell layer were combined and analyzed on 4-15% gradient SDS-PAGE in the presence of 1mM DTT. After electrophoresis, the gels were exposed to X-ray film for 24-48h. For quantitative assay, autoradiograms were scanned with a densitometer and elastin synthesis relative to total protein synthesis was determined.

Total RNA was extracted from cultured cells as described previously and stored at the concentration of 1μg/μl at -80°C. RNA was denatured in 1M glyoxal at 50°C for 1h and resolved by electrophoresis on 1% agarose gel (10μg RNA/lane). Then, blotted to nylon membrane filters. The filters were hybridized in the solution (50% deionized formamide, 5×Denhardt's solution, 0.1% SDS, 1×SSC and 100μg/ml tRNA) at 42°C for 24h with appropriate 32P-labeled cDNA probes. The cDNAs for chicken elastin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin and c-myc were labeled with [32P]dCTP to a specific activity of ~10⁸cpm/μg DNA using multiprime DNA labeling system (Amersham). Filters were washed with two changes of 2 x SSC/0.1% SDS at room temperature for 30 min, then with two changes of 0.1 x SSC/0.1% SDS for 30 min. Filters were air-dried and autoradiographed. The autoradiograms were scanned with a densitometer.

Potent growth-stimulating factors for VSMCs such as EGF, angiotensin II, high K+ concentration or TPA were found to inhibit elastin synthesis in VSMCs, and minoxidil, heparin and retinoic acid which are potent inhibitors for VSMC proliferation were shown to stimulate elastin synthesis (Table 1). These data strongly suggest that elastin expression and VSMC proliferation are coupled tightly and inversely: Potent stimulators of cell proliferation may potentially inhibit elastin expression and potent inhibitors for cell proliferation can stimulate elastin expression. The effect of heparin or TPA on elastin expression and VSMC proliferation was found to be mediated by the modulation of protein kinase C (PKC) activity using various antagonists of kinases including H-7, W-7 and HA1004.

### Table 1 Correlation between Elastin Expression and SMC Proliferation by Various Factors

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<tr>
<th>Cell Proliferation</th>
<th>Elastin Expression</th>
<th>PKC Activity</th>
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<td>Minoxidil</td>
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### Expression of elastin in a serum-deprived quiescent and serum-induced proliferating cells

Cells were considered to become quiescent (G0) by 48h of serum deprivation, based on the cessation of DNA synthesis and a reduction in c-myc mRNA expression (Fig 1). Upon readdition of serum, cells left the G0 phase, and DNA synthesis and c-myc mRNA level were stimulated within 24h up to the level of the proliferating state (Fig 1). Under these conditions, the elastin mRNA level was increased 3.5-fold by 48 and 72h of serum deprivation and declined to the level of the proliferative state by 24h of serum addition (Fig 1).

### Expression of elastin in the suspension-arrested cells or during cell cycle

Studies with the growth control systems using a serum deprivation/readdition method in confluent culture cannot properly address the question regarding the relationship present between cell growth and elastin expression since growth factors derived from serum including EGF have been known to control elastin expression. To address this question adequately, smooth muscle cells, anchorage-dependent cells, were made quiescent using a suspension culture system. Suspension of smooth muscle cells in methylcellulose culture for 72h caused the majority of cells to enter the G0 phase based on the cessation of DNA synthesis and a reduction in c-myc mRNA expression. Under this condition, the elastin mRNA level was markedly enhanced by 72h, suggesting that elastin was maximally expressed at a G0 state. This was confirmed by the results obtained from cell synchronization experiments. The cells started to divide at the 10h time point after hydroxyurea treatment and the number of

### Elastin Expression Is Modulated by Cell Proliferative State

To clarify the possibility that the modulation of elastin expression is mediated by the modulation of VSMC proliferation, we studied the effects of the cell proliferative state on elastin expression using three different culture systems: Deprivation of serum growth factors in confluent cells, suspension culture independent of serum growth factors or adhesion, and the cell synchronization with double thymidine treatment.
Fig 1 Expression of elastin, c-myc and GAPDH mRNAs in smooth muscle cells entering into a quiescent and proliferative state with deprivation and readdition of serum. Confluent cells in 10% FBS medium were shifted to 0.5% FBS medium for 24, 48 and 72h and placed again in the medium containing 10% FBS for 24h. RNA was isolated from the cells at the indicated times and subjected to Northern blot analysis. Cell number and thymidine incorporation were determined at the termination of incubation.20

cells increased 2-fold over the basal level at the 11h time point (Fig 2). Incorporation of thymidine was sharply elevated at the 3h time point and rapidly declined to basal level at the 6h time point, indicating that the cells were synchronized at the G1/S-interphase at zero-time and were in the S and M phases at the 3 and 10–11h time points respectively (Fig 2). The elastin mRNA level as measured by Northern blot hybridization was found to be unaltered during the G1/S phases and to have declined at the G2/M phase (Fig 2a). The elastin mRNA level of the cells which were brought to the G0 phase by the treatment of density-arrested cells with serum deprivation for 72h (lane D) or by the suspension culture in 10% methylcellulose for 72h (lane S) was simultaneously determined in the same filters. The elastin mRNA level in the G0 phase appeared to be maximally expressed compared with that in the G1-S-G2-M phases. The collagen mRNA level in the G0 phase was slightly higher than that in any phases of the cell cycle. These results suggest that the elastin expression and cell cycle are closely regulated in VSMCs and that the elastin gene could be a cell cycle-related gene. The reason why the expression of elastin, an extracellular matrix protein, is regulated by the cell cycle is unclear at present. Under normal conditions, the vast majority of VSMCs in the medial layer of the artery, elastin-rich tissue, are believed to be in a stationary phase ("contractile" state) under constraints of the extracellular matrices.1 The proliferation of VSMCs have been reported to be selectively inhibited on the α-elastin-coated dishes but not on the type I- and fibronectin-coated dishes.21 This suggests...
that VSMCs can express elastin its maximum level in the medial layer and elastic fibers accumulated in the medial layer, in turn, can hinder the rapid proliferation of VSMCs. Thus, preferential expression of elastin at the G0 phase appears to be regulated, in part, by the elastin molecule present in the extracellular space.

**Cell Proliferation Is Regulated by Elastin Fragments**

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.\(^{23-27}\) VGVAPG is a hexapeptide repeated multiple times in the human, bovine and procine elastin molecules but not present in chicken elastin molecule.\(^{23-27}\) This sequence is active as a chemoattractant for monocytes,\(^{28}\) elastin-producing fibroblasts\(^{29}\) and tumor cells,\(^{30}\) and modulates PKC activity in lung carcinoma cells.\(^{31}\) In this studies, we synthesized a pentapeptide VPGVG which is present in chicken elastin molecule and repeats multiple times.

The elastin peptides, VPGVG monomer and polymer (Fig 3c and e) enhanced VSMC proliferation (1.5-fold) to the same extent as TPA (1.6-fold) (Fig 3a). The peptides, monomer and polymer of VAPGVG, showed no significant effect on cell proliferation (Fig 3d 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 3b). Since both VPGVG monomer and polymer showed an enhanced effect on cell proliferation to the same extent, further experiments were performed using VPGVG monomer alone and VAPGVG as a control. Elastin synthesis was inhibited by the treatment with VPGVG dose-dependently. Relative elastin synthesis measured by autoradiograms demonstrated that maximum inhibition of one third of control was achieved at the concentration of 10\(^{-5}\)M during 48h treatment (Fig 4). In contrast, VAPGVG exhibited no significant effect on elastin synthesis (not shown).

The results demonstrated that exogenously added elastin fragment, VPGVG, stimulated cell proliferation and, at the same time, inhibited elastin expression. The modulations of elastin expression was found to be controlled by cell growth state as has been described at section II. Therefore the affected elastin expression by elastin fragment may be related to its stimulatory effect on cell proliferation. The inhibition of elastin expression by elastin fragments may reflect the negative feed-back regulatory mechanism by which elastin synthesis is controlled under normal and the diseased states. The modulation seems to be reasonable because excess elastin fragments probably generated from accumulated elastic fibers in the elastogenic tissues inhibit elastin synthesis to

![Fig 3](image1.png)  
**Fig 3** Effect of TPA and various elastin peptides on cell proliferation. Quiescent cultures were treated with TPA (a) or various elastin peptide (b-f) at the concentrations indicated for 48h. At the termination of incubation, cells were trypsinized and the numbers of cells were counted. Values are mean ± deviations obtained from duplicate experiments.\(^{22}\)

![Fig 4](image2.png)  
**Fig 4** Effect of elastin peptide on elastin synthesis. Cell were treated with VPGVG for 24h at the concentration of 0, 10\(^{-8}\), 10\(^{-7}\) and 10\(^{-6}\)M. Proteins from medium (a) and cell layer (b) were extracted and resolved on 4–15% SDS-PAGE followed by autoradiography. Elastin synthesis relative to total protein synthesis was estimated from autoradiograms of medium and cell layer fractions (right panel).\(^{22}\)
balance the normal metabolism of elastin.

The modulations were specifically found in the sequences VPGVG not in VAPGVG. 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, suggesting that VPGVG plays an essential role in elastin metabolism in normal and diseased states.

References

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