Cell cycle-dependent regulation of elastin gene in cultured chick vascular smooth-muscle cells

Hiroshi WACHI,† Yoshiyuki SEYAMA,† Saburo YAMASHITA† and Shingo TAJIMA*‡

*Department of Dermatology, Keio University School of Medicine, Shinjuku-ku, Tokyo 160, Japan, and †Department of Clinical Chemistry, Hoshi College of Pharmacy, Shinagawa-ku, Tokyo 142, Japan

A study was made of the relationship between elastin expression and the proliferative state of chick vascular smooth-muscle cells. Confluent cells of primary culture brought to a quiescent state by the deprivation of serum for 72 h exhibited a 5-, 3.5- and 2-fold increase in elastin synthesis, elastin mRNA level and transcriptional activity of elastin gene respectively over those in the proliferative state. On re-addition of serum in serum-deprived culture, cells started to proliferate, and elastin synthesis, its mRNA level and transcription of the gene decreased to the level of a proliferative state within 24 h, indicating that elastin expression in smooth-muscle cells was controlled by their growth states at least in part at a transcriptional level. A comparable

increase in elastin mRNA level was observed when the cell growth was arrested by suspension culture for 72 h. When the cells were synchronized at the G1/S phase with thymidine/hydroxyurea treatment, elastin expression at the G1/S phase was greater than that at the G2/M phase during cell cycling. Elastin mRNA level at the G0 phase brought about by serum-deprivation or suspension culture predominated over that at the G1/S phase during cell cycling. These results indicate that gene expression of elastin and cell cycle are tightly coupled, which is independent of the presence of serum or adhesive state, and that elastin expression could be a biochemical marker for the growth states of smooth-muscle cells.

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 [1,2]. Smooth-muscle cell proliferation in normal circumstances has been reported to be regulated by many mitogens, including epidermal growth factor (EGF) [3,4], angiotensin II [5], platelet-derived growth factor [6] and transforming growth factor β (TGF β) [7].

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 [8]. The synthesis of elastin has been demonstrated to be regulated by many growth factors or cytokines; TGF β [9], insulin-like growth factor [10], EGF [4] and interleukin 1 [11]. These potent regulators for elastin synthesis play a key role in elastogenesis during atherosclerosis.

In this study, we evaluated 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 cell synchronization.

MATERIALS AND METHODS

Cell culture

VSMCs were isolated from aortas of 20-day-old chick embryos by a serial enzyme digestion (0.05 % bacterial collagenase and 0.025 % pancreatic elastase) [12]. The cells were plated at a density of 2.2×10^5 cells/cm² in 35- or 100-mm-diam. tissue-culture dishes (Falcon) and cultured in Dulbecco's modified

Eagle's medium (DMEM) supplemented with sodium bicarbonate (3.7 g/l) and 10 % (v/v) fetal bovine serum (FBS) in a humidified atmosphere of 5 % CO₂ in air at 37 °C. 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 or 72 h (quiescent cells). To bring the cells to proliferative state, quiescent cultures were subsequently placed in 10 % FBS medium for 6 or 24 h (proliferating cells). For suspension-arrested experiments, cells were grown to ~ 70 % confluence and treated with trypsin (0.05 %), then suspended in 10 % (w/v) methylcellulose (4000 centipoises, Sigma) dissolved in DMEM containing 10 % FBS at a density of 1.5×10^5 cells/ml [13] and cultured for 24, 48 and 72 h at 37 °C. At the termination of culturing, cells were harvested by centrifugation at 10000 *g* for 30 min at room temperature, then washed with PBS twice.

Metabolic labelling and elastin synthesis

The cells at a quiescent or proliferating state were labelled with [3,4-³H]valine (20 μ Ci/ml) (2.1 TBq/mmol, Amersham) for 6 h in valine-free DMEM (Gibco) supplemented with 0.5 % FBS for quiescent cells or 10 % FBS for proliferating cells. The protein in the medium was precipitated with ammonium sulphate (176 mg/ml) in the presence of protease inhibitor cocktail containing 1 mM each of EDTA, *N*-ethylmaleimide (NEM) and PMSF. Proteins were extracted from cells with 0.5 M acetic acid containing protease inhibitor cocktail and precipitated with ammonium sulphate (176 mg/ml) after neutralization. Proteins in the medium and cell layer were combined and analysed on 4–15 % gradient SDS/PAGE in the presence of 1 mM dithiothreitol (DTT). After electrophoresis, the gels were stained, immersed in 1.1 M sodium salicylate for 30 min [14], dried *in*

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EGF, epidermal growth factor; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NEM, *N*-ethylmaleimide; NP-40, Nonidet P-40; SSC, 0.15 M sodium chloride and 0.015 M sodium citrate; TGF β , transforming growth factor β ; VSMC, vascular smooth-muscle cell.

‡To whom correspondence should be addressed.

vacuo and exposed to X-ray film (Fuji RX) for 24–48 h. The amounts of radioactivity applied to SDS/PAGE were normalized to the cell number. For quantitative assay, autoradiograms were scanned with a densitometer (Cliniscan, Helena Laboratories), Beaumont, TX, U.S.A. and elastin synthesis relative to total protein synthesis was determined [4].

RNA hybridization analysis

Total RNA was isolated from the cultured cells with guanidine thiocyanate according to the published procedure [15] and adjusted to a concentration of 1 µg/ml and stored at -80°C . Ten micrograms of RNA was denatured in 1 M glyoxal/10 mM phosphate buffer, pH 6.5, at 50°C for 1 h and resolved on 1% agarose gel electrophoresis. RNA was then blotted on to nylon membrane (Pall Biosupport), immobilized with UV cross-links (XL1500, Spectronics Corp.), and prehybridized in a solution (50% deionized formamide, 5× Denhardt's solution, 0.1% SDS, 5× SSC and 100 µg/ml tRNA) at 42°C for 24 h (SSC: 0.15 M sodium chloride/0.015 M sodium citrate). Hybridization was performed for 24 h using the same buffer containing the appropriate ^{32}P -labelled cDNA probes. The cDNAs used here were chick elastin (pTE2) [16], $\alpha_1(\text{I})$ collagen (pCG45) [17], β -actin (pA1) [18], c-myc [19] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [20] and were labelled with [α - ^{32}P]dCTP (110 TBq/mmol, Amersham) to a specific radioactivity of $\sim 10^8$ c.p.m./µg of DNA using the multiprime DNA labelling system (Amersham). Filters were washed with two changes of 2× SSC/0.1% SDS and 0.1× SSC/0.1% SDS at room temperature for 30 min, then subjected to autoradiography. The autoradiograms were quantified by scanning densitometry. The blots were used several times after washing with 1% SDS at 100°C for 15 min.

Nuclear run-on assay

The assay was performed essentially as described previously [21]. Briefly, cells were trypsinized and lysed with a buffer [10 mM Tris/HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl_2 and 0.03% Nonidet P-40 (NP-40)] for 5 min at 4°C , and nuclei were precipitated by centrifugation at 500 g for 5 min at 4°C . The nuclei were resuspended in the same buffer devoid of NP-40 and re-centrifuged at 500 g for 5 min. The nuclei were resuspended in a storage buffer (50 mM Tris/HCl, pH 8.0, 40% glycerol, 5 mM MgCl_2 and 0.1 mM EDTA), from which an aliquot was taken for nuclei count, and stored at -80°C at a concentration of 10^7 nuclei/100 µl. The nuclei (10^7 /100 µl) were mixed with an equal volume of a solution containing 10 mM Tris/HCl, pH 7.4, 5 mM MgCl_2 , 300 mM KCl, 200 units of RNase inhibitor, 2 mM DTT, 0.5 mM each of ATP, CTP, GTP and [α - ^{32}P]UTP (120 TBq/mmol, Amersham) and incubated for 20 min at 30°C . The reaction was stopped by DNase I (Epicentre Technology Co.; 10 units/100 ml) and the sample was deproteinized with proteinase K digestion (Sigma) (100 µg/ml) for 30 min at 42°C . RNA was isolated with phenol/chloroform and precipitated with ethanol. The radioactive incorporation of [^{32}P]UTP into RNA showed a linear correlation with the input number of nuclei between 10^6 and 10^8 . An equal radioactivity of RNA (5×10^6 c.p.m.) was hybridized with cDNAs in a hybridization solution (6× SSC, 0.5% SDS, 5× Denhardt's solution, 100 µg/ml salmon sperm DNA and 50 µg of yeast tRNA) for 48 h at 65°C . DNAs used in this experiment were chicken elastin (pTE2) [16], $\alpha_1(\text{I})$ (pCG45) [17], β -actin (pA1) [18], GAPDH [20] cDNAs and plasmid pBR322 DNA which were isolated and purified as described elsewhere [22]. One microgram of each DNA was blotted on to nitrocellulose filters (Schleicher & Schuell) using

a vacuum manifold (Bio-Rad), denatured with 0.1 M NaOH, neutralized and baked at 80°C for 2 h. The filters were washed with 2× SSC four times for 30 min at 65°C and two times for 30 min at room temperature, then air-dried and autoradiographed. The autoradiograms were scanned with a densitometer.

Introduction of synchrony with thymidine/hydroxyurea block

Cells were plated at a density of 1.1×10^5 cells/cm² in 35-mm-diam. dishes and cultured in DMEM containing 10% FBS. After 24 h, cells were treated with 2.5 mM thymidine for 24 h which caused the accumulation of dTTT and blockage of DNA synthesis. The treatment induces the cells at other phases than S phase to run towards G1/S-interphase. The medium containing excess thymidine was removed and the cells were washed with PBS three times, then cultured for 10 h in fresh 10% FBS medium to run again the cell cycle from G1/S-interphase or S phase. The second DNA block with hydroxyurea (10 mM) treatment for 16 h was performed at the time just before the running cells entered into the second S phase. Cells were released from the second block by three washes with PBS, then incubated in 10% FBS medium for 0, 3, 6, 8, 10 and 11 h to initiate synchronous growth [23]. At the termination of incubation, cells were labelled with [methyl- ^3H]thymidine (1 µCi/ml) for 30 min. Incorporation of thymidine and cell numbers were determined as described above.

RESULTS

Expression of elastin in serum-deprived quiescent and serum-induced proliferating cells

Cells were considered to become quiescent (G0) by 48 h of serum deprivation, based on the cessation of DNA synthesis and a reduction in c-myc mRNA expression (Figure 1) [24,25]. Upon re-addition of serum, cells left the G0 phase [24], and DNA synthesis and c-myc mRNA level were stimulated within 24 h up

Figure 1 Expression of elastin, c-myc and GAPDH mRNAs in smooth-muscle cells entering into a quiescent and proliferative state with deprivation and re-addition of serum

Confluent cells in 10% FBS medium were shifted to 0.5% FBS medium for 24, 48 and 72 h and placed again in the medium containing 10% FBS for 24 h. RNA was isolated from the cells at the indicated times and subjected to Northern blot analysis (a). Cell number and thymidine incorporation were determined at the termination of incubation (b).

Figure 2 Expression of collagen, elastin and β -actin mRNAs in the early phase of quiescent or proliferating cells

Confluent cells in 10% FBS medium were shifted to 0.5% FBS medium for 0, 3, 6, 12, 24 and 48 h and placed in 10% FBS medium for 6 and 24 h. RNA was isolated and determined as described in Figure 1.

Figure 3 Nuclear run-on transcription assay of collagen, elastin and β -actin genes

Nuclei were isolated using the NP-40 lysis method from quiescent or proliferating cells. *In vitro* transcription run-on assays were performed and 32 P-labelled run-on transcripts were hybridized to collagen, elastin, β -actin and GAPDH cDNA clones and PBR322 plasmid DNA immobilized on nitrocellulose filters. The filters were washed and subjected to autoradiography (a). The autoradiograms were scanned with a densitometer and transcriptional activities of the genes were determined. Values are the mean $\pm$ range from duplicate experiments.

to the level of the proliferating state (Figure 1). Under these conditions, the elastin mRNA level was increased 3.5-fold by 48 and 72 h of serum deprivation and declined to the level of the proliferative state by 24 h of serum addition (Figure 1). To analyse the time-lapse and reversibility of the increase in elastin

Figure 4 Synthesis of elastin in the smooth-muscle cells entering into a quiescent or proliferating state

Cultures of confluent cells in 10% FBS medium were shifted to 0.5% FBS medium for 48 h, then placed again in the medium containing 10% FBS for 24 h. Cells at the periods of 0 (lane 1), 12 (lane 2), 24 (lane 3) and 48 h (lane 4) after shifting to 0.5% FBS medium and 24 h after re-addition of 10% FBS medium (lane 5) were labelled with [3 H]valine for the last 6 h. Labelled proteins in cultured medium and cell layers were combined and subjected to 4–15% SDS/PAGE followed by autoradiography (a). Elastin synthesis relative to total protein synthesis was measured by scanning densitometry (b). Values are the mean $\pm$ range from duplicate experiments.

mRNA level in detail, mRNA levels of elastin as well as collagen and β -actin isolated from the cultures at 0, 3, 6, 12, 24 and 48 h under the serum-deprivation condition, and at 6 and 24 h under the serum re-addition condition, were determined. Elastin mRNA started to increase at 24 h of serum deprivation and reached a 3.5-fold increase by 48 h. Re-addition of serum to the culture of serum-deprived quiescent cells resulted in a decline in the mRNA level for elastin to the level of the initial stage by 24 h (Figure 2). The collagen mRNA level showed a slight increase (1.7-fold after 48 h of serum deprivation) as the cells became quiescent and returned to the initial level by 24 h of the re-addition of serum (Figure 2). The level of β -actin mRNA exhibited a slight reduction (half of zero time) by 12 h after serum deprivation and was restored to its initial level by the re-addition of serum (Figure 2).

Nuclear run-on assays demonstrated that the transcriptional activity of the elastin gene was stimulated 2-fold after 72 h of serum deprivation. The enhanced transcription was reduced to the initial level by re-stimulation with serum. The rates of transcription of β -actin, collagen and GAPDH genes were essentially constant throughout the serum deprivation and re-addition (Figures 3a and 3b).

Synthesis of tropoelastin relative to total protein synthesis was increased 5-fold after 24 or 48 h of serum deprivation and returned to the basal level after 24 h of serum re-addition (Figures 4a and 4b).

Figure 5 Expression of collagen, elastin, β -actin and c-myc mRNAs in smooth-muscle cells entering into the quiescent state by suspension culture

Exponentially growing cells were trypsinized and transferred to the suspension condition containing 10% methylcellulose in the presence of 10% FBS for 72 h. RNA was extracted from the cells before (lane 1) and after 24 (lane 2), 48 (lane 3) and 72 h (lane 4) of suspension cultures and hybridized with ^{32}P -labelled collagen, elastin, β -actin and c-myc cDNAs (a). DNA synthesis at the same time points was measured as described in the Materials and methods section (b).

Elastin mRNA levels in the suspension-arrested cells

Reduction of DNA synthesis and the c-myc mRNA level during the suspension culture for 72 h in the presence of 10% FBS suggested that the cells entered into the quiescent (G0) state by 72 h (Figures 5a and 5b). Under this condition, the collagen mRNA level exhibited a slight increase by 72 h and the elastin mRNA level was stimulated 4-fold by 72 h. In contrast, the β -actin mRNA level was reduced by one-half as has been reported previously [26] (Figure 5a).

Elastin mRNA level during cell cycle

The cells started to divide at the 10 h time point after hydroxyurea treatment and the number of cells increased 2-fold over the basal level at the 11 h time point (Figure 6b). Incorporation of thymidine was sharply elevated at the 3 h time point and rapidly declined to the basal level at the 6 h 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 10–11 h time points respectively (Figure 6b). 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 (Figure 6a). The elastin mRNA levels of the cells which were brought to the G0 phase by the treatment of density-arrested cells with serum deprivation for 72 h (lane D) or by the suspension culture in 10% methylcellulose for 72 h (lane S) were simultaneously determined in the same filters. The elastin mRNA level in the G0 phase appeared to be maximally expressed

Figure 6 Expression of elastin, collagen and β -actin mRNA in synchronized cells

Cells were treated with 2.5 mM thymidine for 24 h and later with 1 mM hydroxyurea for 16 h. After the treatment, cells were incubated for 0, 3, 6, 8, 10 and 11 h in 10% FBS DMEM. At the termination of incubation, RNA was extracted from cells and elastin, collagen and β -actin mRNA levels were assayed by Northern blot analysis. RNAs at the G0 phase by the treatment of density-arrested cells with serum deprivation for 72 h (lane D) or by the suspension culture in 10% methylcellulose for 72 h (lane S) were simultaneously assayed (a). Cells were labelled with $[^3\text{H}]$ thymidine for the final 30 min and the number of cells and thymidine incorporation were determined after trypsin treatment (b).

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 phase of the cell cycle. In contrast, β -actin mRNA was constitutively expressed throughout the cell cycle including the G0 phase (Figure 6a).

DISCUSSION

Studies with the growth control systems using a serum deprivation 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 [4]. To address this question adequately smooth-muscle cells, anchorage-dependent cells, were made quiescent using a suspension culture system in the presence of serum. Suspension of smooth-muscle cells in methylcellulose culture for 72 h 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 72 h, suggesting that elastin was maximally expressed at a G0 state. This was confirmed by the results obtained from cell synchronization, in which the expression of elastin mRNA in the G0 phase induced by serum deprivation or suspension culture appeared to predominate over the G1, S, G2 and M phases, unlike collagen and β -actin mRNAs. 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 [1,27,28]. The proliferation of VSMCs has been reported to be selectively inhibited on the α -elastin-coated dishes but not on the type I- and fibronectin-coated dishes [29]. This suggests that VSMCs can express elastin at its maximum level in the medial layer, and elastic fibres 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.

The data presented here demonstrate that expression of elastin by VSMCs is regulated by the cell proliferative state. Indeed many mitogenic factors for VSMCs like EGF [4], high K ion [30] and angiotensin II [31] have been reported to be able to suppress elastin expression and to stimulate DNA synthesis. Furthermore, cyclic nucleotide 8-bromo-cyclic GMP, which accelerates Ca^{2+} efflux in cultured VSMCs by stimulating the Ca^{2+} pump [32], has been reported to enhance elastin synthesis [33]. We have recently shown that elastin expression in the proliferating VSMCs was specifically stimulated by H-7, a potent inhibitor for protein kinase C, but not by H-89 and W-7, inhibitors for A kinase and calmodulin kinase (H. Wachi, Y. Seyama, S. Yamashita and S. Tajima, unpublished work). These previous reports and our data strongly suggest that elastin expression and VSMC proliferation are coupled tightly and inversely: potent stimulators of cell proliferation can inhibit elastin expression and potent inhibitors for cell proliferation can stimulate elastin expression through their modulation of cell proliferation. All these modulations of elastin expression will be explained by the cell-cycle dependency of elastin expression. Elastin expression thus will be a good biochemical marker to specify the growth state of chick VSMC, although we do not know whether or not these modulations can be extended to other mammalian VSMCs.

We used the cells plated at an initial density of $2.2 \times 10^5/\text{cm}^2$ in these experiments. Since it has been reported that the difference in the initial plating density influences elastin synthesis when the cells reach confluent density [34], studies on the effect of different plating density must be elucidated.

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