Tropoelastin-derived Degradation Products Downregulate Elastin Expression in Vascular Smooth Muscle Cell in Culture

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Abstract: To define the physiological role of elastin peptides in elastin metabolism in vascular wall, effects of tropoelastin-derived fragments produced by pancreatic elastase digestion on elastin expression were studied in chick aortic smooth muscle cells in culture. Treatment of [3H] valine-labeled medium proteins with 0.1 ng/ml pancreatic elastase for 6 h resulted in the specific degradation of newly-synthesized tropoelastin. When cultures were treated with the medium containing tropoelastin-derived fragments, elastin synthesis and its mRNA level were lowered to one half of basal level. The results provide a direct evidence that elastin peptides may negatively autoregulate elastin expression in vascular smooth muscle cells.

Keywords: elastin, elastase, elastin peptide, cell proliferation

INTRODUCTION

Elastin is a major component of arterial wall and confers elasticity to the tissue.1-3 Elastin is produced by smooth muscle cells as a form of tropoelastin with the molecular weight of 68 kDa. The expression of elastin by smooth muscle cells has been demonstrated to be regulated by a number of growth factors and cytokines.4-6 Elastase has been demonstrated to be present in vessel wall4-6 and to modulate the functions of aortic smooth muscle cells.5-7 We have previously demonstrated that synthetic elastin hexapeptide VPGVG, enhance the proliferation of smooth muscle cells and reduces elastin expression.8 This autoregulation of elastin expression by synthetic elastin peptide may play an important role in elastin metabolism in normal and elastogenic conditions. In this report, we investigated whether tropoelastin digests produced by pancreatic elastase influence elastin expression in smooth muscle cells.

MATERIALS AND METHODS

Materials
Porcine pancreatic elastase was donated by Eisai Pharmaceutical Co. (Tokyo, Japan). [3, 4-3H] Valine (1.5 TBq/mmol), [35S] methionine (40 TBq/mmol), [α-32P] dCMP (110 TBq/mmol) and [2,3-3H] proline (2.0 TBq/mmol) were purchased from Amersham.

Cell culture and metabolic labeling
Vascular smooth muscle cells were isolated from the aortas of 20-day-old chick embryos by a serial enzyme digestion (0.05% bacterial collagenase and 0.025% pancreatic elastase).9 The cells were plated at a density of 2.2×10⁶ cell/cm² in 35-mm diameter tissue culture dishes (Falcon) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with sodium bicarbonate (3.7 g/L) and 10% 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 otherwise indicated. When the cells reached confluent density, cultures were labeled for 18 h with [3H] valine (50 μCi/ml) in valine-free DMEM (Gibco) or [35S] methionine (100 μCi/ml) in serum-free DMEM. [3H] Valine- and [35S] methionine-labeled culture medium (1 ml) was harvested, placed in test tubes, then incubated with various doses of pancreatic elastase for 24 h at 37°C in the presence or absence of phenylmethylsulfonyl fluoride (PMSF) (2×10⁻⁴ M).7 Reaction was terminated by heating for 2 min at 95°C and the proteins were precipitated with 176 mg/ml ammonium sulfate, then processed to SDS-polyacrylamide gel electrophoresis (see below).

Cultures were incubated in serum-free DMEM for 18 h. The medium was harvested and digest-
ed with elastase (0.1 ng/ml) for 24 at 37°C as described above except that radioactive materials were omitted. The reaction was terminated with $2 \times 10^{-4}$ M PMSF and placed in separate series of smooth muscle cell cultures for 0, 12 and 24 h. The cultures were then labeled with $^{3}$H]proline for the last 12 h of treatment. To assess the effects of elastin degradation products on elastin expression, the cultures were labeled with $^{3}$H]proline for final 12 h of the treatment in DMEM supplemented with cold valine (10 mM).

**SDS-PAGE and fluorography**

The medium was mixed with proteinase inhibitor cocktail (1 mM of each N-ethylmaleimide, PMSF and ethylenediaminetetraacetic acid) and the proteins were precipitated with ammonium sulfate (176 mg/ml). The cell layer was extracted with 0.5 M acetic acid containing proteinase inhibitor cocktail and the resultant supernatant was lyophilized. The proteins from culture medium and cell layer were individually analyzed on 2-15% gradient SDS-PAGE under reducing conditions followed by fluorography. Equal volumes of the ammonium sulfate-precipitated materials were applied to the gels. Tropoelastin synthesis was measured by scanning the fluorograms with a densitometer (Shimazu Co.) and expressed by percentage of total protein synthesis.

**RNA hybridization analysis**

Total RNA was isolated from the cultured cells with guanidinium thiocyanate according to the published procedure, adjusted to a concentration of 1 μg/μl and stored at −80°C. Ten micrograms of RNA were 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 onto nylon membrane (Pall Biosupport), immobilized with UV crosslinker (XL 1500, Spectronics Corp.) and hybridized with $^{32}$P-labeled cDNA probes. The cDNAs used here were chicken elastin (pTE2), chicken proα1 (1) collagen, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNAs, and labeled with $^{32}$P[dCTP to a specific activity of $10^8$ cpm/μg DNA using multiprime DNA labeling system (Amersham). Filters were hybridized with $^{32}$P-labeled probes for 24 h at 42°C in a solution of 50% deionized formamide, 5 × SSC, 5 × Denhardt’s solution, 0.1% SDS and 250 μg/ml sonicated salmon sperm DNA. The filters were washed at a stringency of 0.1% SDS/0.1 × SSC at room

Fig. 1 Degradation of medium proteins by pancreatic elastase. $^{3}$H] Valine (A)- or $[^{35}S]$methionine (B)-labeled medium was treated with 0, 0.01, 0.1 or 1 ng/ml elastase for 24 h at 37°C in the absence (a) or presence (b) of $2 \times 10^{-4}$ M PMSF. The proteins in the medium were resolved on 2-15% SDS-PAGE followed by autoradiography. Arrow indicates migration position of tropoelastin. 0° in A-b indicates vehicle (isopropyl alcohol) alone.
temperature for 30 min × 3 and exposed to X-ray films at −80°C. The autoradiograms were scanned for quantification with a densitometer.

RESULTS AND DISCUSSION

In the absence of PMSF, pancreatic elastase at the doses lower than 0.01 ng/ml had no effect on newly synthesized proteins. At the dose of 0.1 ng/ml elastase showed relatively specific degradation activity to elastin without degrading other proteins. Non-elastin proteins became sensitive to elastase at the dose of 1 ng/ml (Fig. 1A-a). In the presence of 2 × 10⁻⁴ M PMSF, synthesized proteins were resistant to elastase at the doses lower than 0.1 ng/ml but became degraded at the dose of 1 ng/ml (Fig. 1A-b).

To ensure that elastin was specifically digested by elastase at the dose of 0.1 ng/ml as observed by use of [³H]valine, we have done the same experiment using [³⁵S]methionine, an amino acid lacking in elastin molecule. The results showed that elastase at the doses of 0.1 ng/ml in the absence of PMSF had no degrading effect on the proteins other than elastin (Fig. 1B-a). These results indicated that elastase could specifically digest tropoelastin at the dose of 0.1 ng/ml at 37°C for the duration of 24 h.

To assess the effects of elastin degradation products on elastin synthesis, non-radioactive medium which had been cultured for 18 h was treated with 0.1 ng/ml elastase for 24 h at 37°C. The reaction was terminated with 2 × 10⁻⁴ M PMSF and placed in separate series of cultures for 0, 12 and 24 h and then labeled with [³H]proline for the final 12 h of the treatment. In preliminary experiments, 2 × 10⁻⁴ M PMSF exhibited no cellular toxicity based on the unaltered cell number and incorporation of thymidine (not shown). The results indicated that elastin-derived degradation products inhibited the synthesis of tropoelastin to half of control during the duration of 24 h (Fig. 2a and b). Northern blot assay showed that elastin mRNA level was also inhibited to half of control without significant changes of α₁ (I) collagen and GAPDH mRNA levels (Fig. 3a and b).

![Fig. 2](image-url) Effects of the elastase-treated medium on elastin synthesis in cell culture. Non-radioactive culture medium was treated with 0.1 ng/ml elastase for 24 h at 37°C as described in Fig. 1. The reaction was terminated with 2 × 10⁻⁴ M PMSF, placed in the culture for 0, 12 and 24 h and then labeled with [³H]proline for the last 12 h of the treatment. Proteins were extracted from medium or cells and separated by 2–15% gradient SDS-PAGE followed by autoradiography as described in Materials and Methods. Arrows indicate migration position of tropoelastin (a). Elastin synthesis relative to total protein synthesis was measured by scanning the autoradiograms with a densitometer. Values are means from duplicate experiments (b).
We have previously demonstrated that synthetic elastin hexapeptide, VPGVG, enhances the proliferation of smooth muscle cells and reduces elastin expression. However, it was not clear whether or not elastin peptides with multiple repeats of VPGVG produced in vivo during elastolytic conditions, may have such functions on smooth muscle cell phenotypes. We found the condition in which pancreatic elastase could specifically cleave tropoelastin in test tubes and used the reaction mixture to assess the effects of degradation products on elastin expression.

This report may provide a direct evidence that the elastin peptides produced by enzymatic cleavage can negatively autoregulate elastin expression in smooth muscle cell culture.

The amount of elastin peptide present in the culture media in the culture conditions employed here may be estimated ~0.05 μM judging from the intensity of tropoelastin band after SDS-PAGE. The data appear to be compatible to our previous reports that 10^{-7} M elastin peptide VPGVG reduced tropoelastin synthesis by one third of control.8)

REFERENCES


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