Endothelin-1 Down-Regulates Expression of Tropoelastin and Lysyl Oxidase mRNA in Cultured Chick Aortic Smooth Muscle Cells

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Endothelin-1 (ET-1) is known as a potent stimulator of cell proliferation and as a vasoconstrictor. It is believed that ET-1 contributes to the development of arterial diseases such as atherosclerosis. In this study, we demonstrated the expression of tropoelastin and lysyl oxidase (LO) on gene levels as induced by ET-1 in cultured smooth muscle cells (SMCs). ET-1 stimulated cell proliferation in a dose–dependent manner, and the level of this proliferation increased about 1.3-fold at 100 nM of ET-1. ET-1 suppressed the tropoelastin protein synthesis in a dose–dependent and time-dependent manner. In addition, ET-1 dose–dependently suppressed the tropoelastin and LO mRNA expression. The tropoelastin and LO mRNA levels decreased to about half and 4/5, respectively, at 100 nM of ET-1. The inhibition of elastin synthesis was completely prevented by BQ123, an endothelin receptor A (ET_A) antagonist. These results indicate that ET-1 can modulate the tropoelastin and LO mRNA expression *via* an ET_A receptor in cultured SMC and that the regulator for elastin expression may play an important role in elastogenesis and SMC proliferation during the development of atherosclerosis.

Key words —— elastin, lysyl oxidase, endothelin-1, smooth muscle cell, endothelin A receptor

INTRODUCTION

The regulation of cell proliferation is an important event in normal development and in pathological responses to injury. A number of growth factors and cytokines are capable of stimulating proliferation of target cells by activating their receptors. However, the presence of growth factors or cytokines and their receptors is not sufficient to induce cell proliferation. The number of the local environment, extracellular matrix and intracellular interactions can also regulate the response to a given growth factor or cytokine.

Endothelin-1 (ET-1), which is a potent vasoconstrictor, has been isolated from cultured porcine aortic endothelial cells.[1] ET-1 is mainly synthesized and secreted from cultured porcine endothelial cells, and it is known that ET-1 stimulates cell proliferation.[2] The effects of ET-1 are mediated *via* two subtypes of endothelin receptors, endothelin A (ET_A)[3] and endothelin B (ET_B)[4] receptors, which have been cloned and characterized. ET_A membrane receptors have a high affinity for ET-1 and are mainly present in vascular smooth muscle cells in order to mediate vasoconstriction.[3] The ET_A receptor is located predominantly on vascular smooth muscle cells (SMCs). It is a classical heptathelical G-protein coupled receptor that activates phospholipase C to cause hydrolysis of phosphatidyl inositol and generation of cytosolic inositol triphosphate and membrane-bound diacylglycerol. It has been reported that production of ET-1 occurs in atherosclerosis[5,6] and that ET_A receptor antagonists or endothelin-converting enzyme inhibitors suppress the formation of atheroma.[7] These observations suggest the possibility that ET-1 may stimulate cell proliferation and development of atherosclerosis.

The regulation of accumulation of extracellular matrices is fundamental important to mature tissues and to an understanding of connective tissue diseases such as atherosclerosis. Elastin is responsible for the characteristic elastic properties of many tissues including skin, lung and large blood vessels. Elastin is synthesized as a soluble precursor, tropoelastin, by aortic smooth muscle cells. Tropoelastin is associated with microfibrils, such as fibrillin-1, fibrillin-

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2 or the microfibril associated glycoprotein (MAGP), and then cross-links with other tropoelastin molecules by lysyl oxidase (LO) to form elastic fiber. Elastin synthesis is modulated by a number of factors including transforming growth factor β (TGF-β),[8] insulin-like growth factor (IGF),[9] interleukin-1 (IL-1),[10] epidermal growth factor (EGF),[11] glucocorticoid,[12] and c-guanosine 5′-monophosphate (c-GMP).[13]

Abnormal metabolism of elastin has been associated with hypertension[14] and atherosclerosis,[15] and elastin may play a key role in the development of these diseases. However, there is little report about the gene expression concerning the elastic fiber assembly in the development of atherosclerosis. Therefore, in the present study we investigated the tropoelastin and LO gene expression by ET-1 in cultured chick SMCs.

MATERIALS AND METHODS

Materials —— [3,4-^{3}H] Valine (1.5 TBq/mmol) and [α-^{32}P] deoxycytidine 5′-triphosphate (dCTP) (110 TBq/mmol) were supplied by Amersham. ET-1 was purchased from the Peptide Institute, Inc. (Japan), BQ123 was obtained from Alexis, and Dulbecco's modified Eagle's medium (DMEM), valine-free DMEM, fetal bovine serum (FBS), and dialyzed FBS were obtained from Gibco.

Cell Culture and Proliferation —— SMCs were isolated from 20-day-old chick embryonic aortas by serial enzyme digestion with bacterial collagenase (Sigma) and pancreatic elastase (Sigma) as previously described.[16] They were seeded at a density of 2×10^6 cells/35-mm-diameter Petri dish (Falcon Plastics) and grown to 80% of confluence in DMEM supplemented with 10% FBS. The FBS and dialyzed FBS used in this study were not heat-inactivated. SMCs grown to 80% of confluence were incubated for 24 hr in DMEM containing 0.5% dialyzed FBS to induce them to the G_0 phase (quiescent). The quiescent SMCs were cultured for 24 hr in DMEM containing 0.5% dialyzed FBS with various concentrations of ET-1 and then were harvested with 0.25% trypsin. The cell number was determined with a hemocytometer. The data are presented as a means ± standard error (S.E.). Statistically significant differences between groups were analyzed using Student's t test, where $p < 0.05$ was considered statistically significant. Statview software (Abacus, Berkeley, CA, U.S.A.) was used for all statistical work.

Metabolic Labeling and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) —— The quiescent SMCs were treated with various concentrations of ET-1 in DMEM containing 0.5% dialyzed FBS for 24 hr or with 100 nM ET-1 in DMEM containing 0.5% dialyzed FBS for various periods of time. Quiescent SMCs were pretreated with 10 μM BQ123, as an inhibitor of endothelin receptor A (ET_A), in DMEM containing 0.5% dialyzed FBS for 30 min and then treated with a combination of 10 μM BQ123 and 10^{-7} M ET-1 in DMEM containing 0.5% dialyzed FBS for 24 hr. The cells were labeled with 25 μCi/ml of [3,4-^{3}H] valine for the last 6 hr of treatment in the valine-free DMEM. The culture medium was precipitated with ammonium sulfate (176 mg/ml) in the presence of protease inhibitor cocktails [1 mM EDTA, *N*-ethylmaleimide (NEM) and phenylmethylsulfonyl fluoride (PMSF)]. The protein from the medium was resuspended in 35 μl of Laemmli sample buffer containing dithiothreitol (DTT) and incubated at 100°C for 5 min. The samples were electrophoresed on 4–15% SDS-polyacrylamide gels. Gels were then dried and exposed to XAR-5 X-ray film (Eastman Kodak Co., U.S.A.), and then fluorographed and scanned with a densitometer (Cliniscan, Helena Laboratorie, U.S.A.). The amount of samples applied to the gels was normalized according to cell number. The density of elastin bands per total bands was used as a measure of relative synthesis.

Northern Blot Analysis —— Total RNA was isolated from cells according to a previously described procedure[17] and after being adjusted to a concentration of 2 μg/μl, stored at –80°C degree until use. The total RNA was denatured for 1 hr at 50°C in deionized 1 M glyoxal/10 mM phosphate buffer, pH 7.0, and electrophoresed on 1% agarose gel, then was blotted to N^+ nylon filters (Amersham, U.K.). The membranes were hybridized for 18 hr at 42°C to ^{32}P-labeled probes in 50% formamide, 5 × 0.15 M sodium cloride and 0.015 M sodium citrate (SSC), 5 × Denhardt's solution, 0.1% SDS, and 250 μg/ml t-RNA. The following cDNA probes which were radioactively labeled by random priming (Amersham, U.K.) to specific activity of $\sim 10^8$ dpm/μg DNA were used: chicken elastin (pTE2),[18] and β-actin (pA1).[19] 300 bp of Chick LO cDNA[20] were amplified by reverse transcriptase–polymerase chain reaction (RT–PCR) using a sense primer (5′-ACGGACGATAACCCCTACTACAACT-3′) and an anti-sense primer (5′-CGCACTATGTTGTTGGAG-

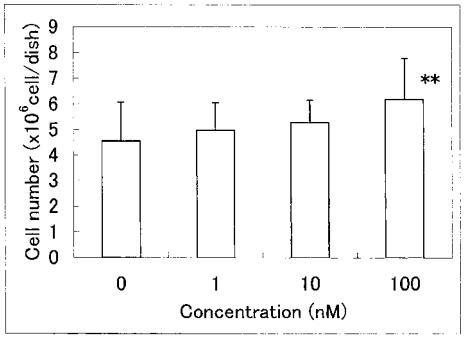

Fig. 1. Effect of Endothelin-1 on Cell Proliferation

SMCs were plated at a density of 2×10^6 cells/35 mm-diameter Petri dish and cultured in DMEM supplemented with 10% FBS. 80% of confluence SMCs were treated for 24 hr with 0, 1, 10, 100 nM of endothelin-1 in FBS-free DMEM, and then were harvested by trypsin. Cell number was counted by Buker-Turk hemocytometer. Each value indicates the means ± S.E. from triplicate experiments; $^{**}p < 0.01$ (comparison with control).

TAATCAG-3′). The filters were washed for 30 min at a stringency of 1 × SSC/0.1% SDS followed by 0.1 × SSD/0.1% SDS and exposed at –80°C to X-ray films (Fuji RX, Japan) with an intensifying screen (Kodak Lanex Regular, U.S.A.). The autoradiograms were scanned with a densitometer. The density of tropoelastin and LO bands was normalized according to β-actin bands as a measure of relative expression.

RESULTS

Cell Proliferation by ET-1

To begin with, we determined the effect of ET-1 on cell proliferation. Quiescent SMCs were exposed to 1–100 nM ET-1 for 24 hr of exhibited proliferation compared to control. The effect was most pronounced at 100 nM ET-1, which resulted in 30% stimulation of proliferation ($p < 0.01$; Fig. 1). These results are similar to those reported for human skin fibroblasts in culture.[21]

Tropoelastin Synthesis by ET-1

The changes in tropoelastin protein synthesis in SMCs brought about by drug treatment were detected by SDS-PAGE following a metabolic labeling assay with [^{3}H] valine. Immunoblotting analysis using monoclonal antibody for tropoelastin revealed that the bands, which indicated with an arrow, are related to tropoelastin.[22] Moreover, we previously reported that bands of tropoelastin were confirmed by incorporations of radiolabeled cysteine and valine, but not incorporations of radiolabeled mannose, glucose, and methionine.[23] Since complete amino acid sequence deduced from chicken cDNA demonstrated that tropoelastin has no sugar moiety nor methionine residue and has two cysteine residues only near the carboxyl-terminal end.[18,24,25] Confluent SMCs were cultured with FBS-free DMEM for 24 hr to induce the cells to enter the G_0 phase,[26] and then the culture medium was replaced with FBS-free DMEM containing 0, 1, 10 or 100 nM of ET-1. After 18 hr, newly synthesized proteins were labeled with [3,4-^{3}H] valine. ET-1 inhibited tropoelastin synthesis in the medium in a dose–dependent manner without changing other secreted proteins, and the level of tropoelastin synthesis was about 50% at a concentration of 100 nM of ET-1 (Fig. 2). We also determined the amount of tropoelastin synthesis induced by ET-1 in the medium in a time-dependent manner. ET-1 inhibited about 25% of tropoelastin synthesis after 6 hr of treatment and 50% after 24 hr of treatment (Fig. 3).

Expression of Tropoelastin and LO mRNA by ET-1

To examine the regulation of tropoelastin and LO mRNA expression by ET-1, confluent cultures of SMCs maintained in DMEM without FBS were incubated with various concentrations of ET-1 for 24 hr. Tropoelastin and LO mRNA levels were estimated by northern blot hybridization. ET-1 reduced elastin and LO mRNA levels in a dose–dependent manner, and the maximal inhibition was detected at a concentration of 100 nM (Fig. 4A). Quantification of tropoelastin and LO mRNA, after correction for β-actin mRNA levels, revealed that the maximal reduction in tropoelastin and LO mRNA to approximately 40% and 80% of the level in untreated control cells occurred after treatment with 100 nM of ET-1 (Fig. 4B).

Effect of ET_A Blockers on ET-1-Induced Elastin Synthesis

In order to determine whether the inhibition of tropoelastin synthesis by ET-1 is dependent on ET_A or not, confluent cultures of SMCs were treated with ET-1 (100 nM) alone or in combination with BQ-123 (10 μM), as a specific inhibitor of ET_A, in DMEM without FBS. In these cells, treatment with BQ-123 alone had no effect on tropoelastin synthesis. However, exposure of SMCs to BQ-123 potently prevented the inhibitory effect of ET-1 on tropoelastin synthesis (Fig. 5).

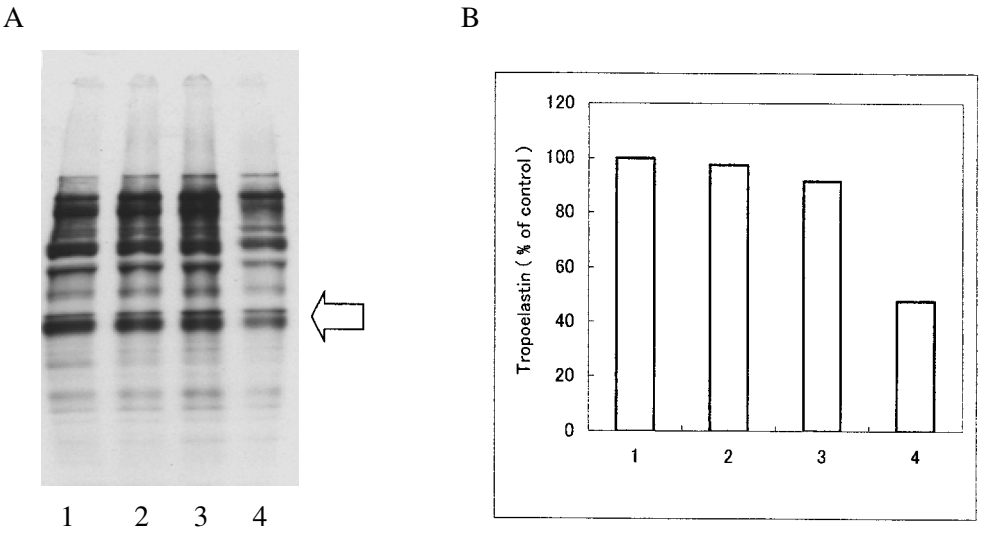

Fig. 2. Effect of ET-1 on Elastin Synthesis in Smooth Muscle Cells

(A), SMCs were treated for 24 hr with 0 (lane 1), 1 (lane 2), 10 (lane 3), 100 nM (lane 4) of ET-1 in FBS-free DMEM, then labeled with [^{3}H] valine for the last 6 hr of treatment. The proteins from culture medium analyzed by 2–15% SDS-PAGE, and visualized by fluorography. (B), The arrow indicates the position of tropoelastin. The fluorograms were quantitated with a scanning densitometer. Each value indicates the average from triplicate experiments.

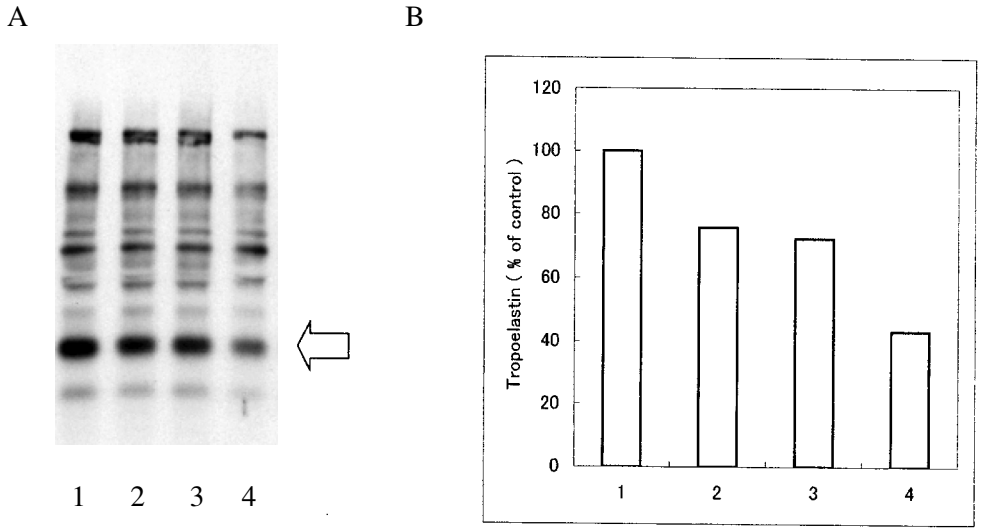

Fig. 3. Effect of ET-1 on Elastin Synthesis in Smooth Muscle Cells

(A), SMCs were treated for 0 (lane 1), 6 (lane 2), 12 (lane 3) and 24 hr (lane 4) with 100 nM endothelin-1 in FBS-free DMEM, then labeled with [^{3}H] valine for the last 6 hr of treatment. The proteins from culture medium analyzed by 2–15% SDS-PAGE, and visualized by fluorography. (B), The arrow indicates the position of tropoelastin. The fluorograms were quantitated with a scanning densitometer. Each value indicates the average from triplicate experiments.

DISCUSSION

SMC proliferation is a key event in the development of atherosclerosis. Several studies have revealed that elastin synthesis is inversely related to cell proliferation. It has been reported that potent stimulators of cell proliferation, such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA),[27] angiotensin II[28] or EGF,[11] inhibit elastin synthesis, and potent inhibitors of cell proliferation, such as minoxidil,[29] retinoic acid[30] or heparin,[31] stimulate elastin synthesis. In this study, our results show that ET-1 stimulates SMC proliferation and reduces tropoelastin synthesis in mRNA levels as previously reported.

ET-1 reduces tropoelastin and LO mRNA expres-

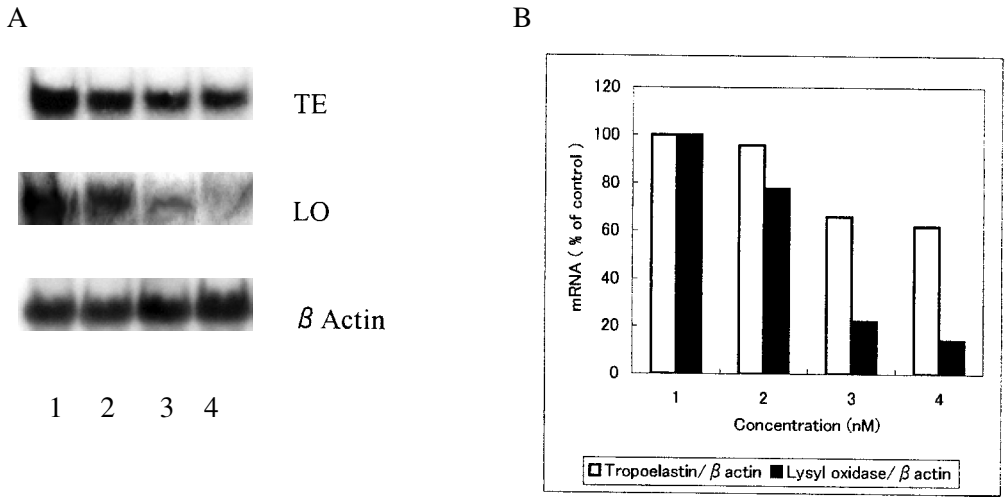

Fig .4. Effect of ET-1 on Tropoelastin (TE), LO and β-Actin mRNA Levels in SMCs

(A), RNA was extracted from SMCs treated for 24 hr with 0 (lane 1), 1 (lane 2), 10 (lane 3), 100 nM (lane 4) of ET-1. Ten micrograms RNA was resolved on 1% agarose gel electrophoresis, blotted onto membranes and hybridized with ^{32}P-labeled chick elastin, LO and β-actin cDNA probes. The filters were washed and visualized by autoradiography. (B), The autoradiograms were quantitated with a scanning densitometer. Open bars represent the quantitative tropoelastin mRNA. Black bars represent the quantitative LO mRNA. Each value indicates the average from triplicate experiments.

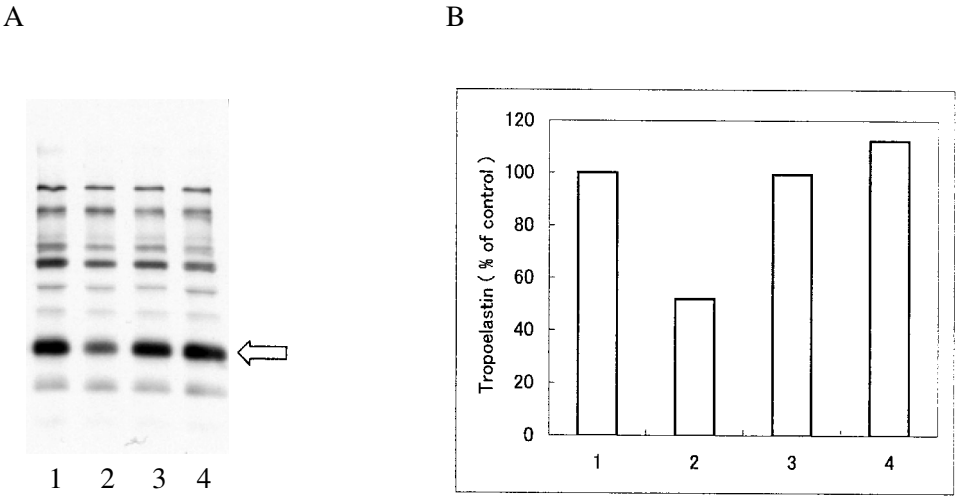

Fig. 5. Effect of BQ123 on ET-1-Mediated Elastin Suppression

(A), SMCs were treated with vehicle (lane 1), 100 nM of ET-1 (lane 2), 10 μM of BQ123 (lane 3) or combination of 100 nM of ET-1 and 10 μM of BQ123 (lane 4) for 24 hr in the absence of FBS, and labeled [^{3}H] valine for the final 6 hr. The proteins from culture medium analyzed by 2–15% SDS-PAGE, and visualized by fluorography. (B), The arrow indicates the position of tropoelastin. The fluorograms were quantitated with a scanning densitometer. Each value indicates the average from triplicate experiments.

sion, and BQ 123, a specific ET_A receptor antagonist,[32] can effectively prevent the effect of ET-1 on tropoelastin synthesis. ET-1 binds to ET_A receptors on the cell surface, and these receptors are classical heptathelical G-protein coupled receptors that activate phospholipase C to cause hydrolysis of phosphatidyl inositol and generation of cytosolic inositol triphosphate and membrane-bound diacylglycerol, which accelerate protein kinase C (PKC) activity and intracellular Ca^{2+} concentration.[33] It has been reported that a PKC activator, such as TPA, reduces elastin expression by a posttranscriptional mechanism. It has also been postulated that TPA may control the tropoelastin mRNA *via* unique *cis*-acting sequences of the 3′ untranslated region (3′UTR).[27] We have demonstrated that ET-1-induced reduction of elastin expression is mediated by the activation of PKC activity *via* ET_A receptors.

LO is a key participant in the accumulation of insoluble fibers of elastin and collagen by virtue of

its role in the initiation of the covalent cross-linkages between and within individual molecules comprising these fibers. This enzyme oxidizes specific lysine residues within these matrix molecules into peptidyl-α-aminoadipic-δ-semialdehyde. Subsequent spontaneous condensations of the aldehyde residues produce the cross-linkages that account for the stability of these fibrous proteins.[34] The expression of the LO gene is regulated at transcriptional or post-transcriptional levels by a number of agents or conditions, or both, relevant to the normal and/or diseased arterial wall. LO expression is significantly up-regulated by TGF-β1,[35,36] platelet-derived growth factor (PDGF)[37] and cAMP.[38] It has been reported that cAMP inhibits the proliferation of vascular smooth muscle cell (VSMC).[39] LO expression is significantly stimulated upon reduction of SMC proliferation in culture either by serum starvation or by the addition of TGF-β1.[35] In the present study, ET-1 inhibited LO mRNA expression in a dose–dependent manner. Together with these reports, our results may suggest that modulation of LO mRNA expression is inversely associated with cell proliferation.

It has been reported that amounts of desmosine or isodesmosine, which are cross-linking amino acids in elastic fiber, decrease in human atherosclerotic plaques.[40,41] ET-1 is known as an activator of atherosclerosis. Therefore, the results of this paper suggest that inhibition of tropoelastin and LO mRNA expression by ET-1 may cause inhibition of the elastin cross-linking processes in the aorta and result in a destabilization of the aortic wall such as occurs in the disease of atherosclerosis.

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REFERENCES

1) Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* (London), **332**, 411–415.
2) Alberts, G. F., Peifley, K. A., Johns, A., Kleha, J. F. and Winkles, J. A. (1994) Constitutive endothelin-1 overexpression promotes smooth muscle cell proliferation via an external autocrine loop. *J. Biol. Chem.*, **269**, 10112–10118.
3) Arai, H., Hori, S., Aramori, I., Ohkubo, H. and Nakanishi, S. (1990) Cloning and expression of a cDNA encoding an endothelin receptor. *Nature* (London), **348**, 730–732.
4) Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K. and Masaki, T. (1990) Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature* (London), **348**, 732–735.
5) Lerman, A., Edwards, B. S., Hallett, J. W., Heublein, D. M., Sandberg, S. M. and Burnett, J. C., Jr. (1991) Circulating and tissue endothelin immunoreactivity in advanced atherosclerosis. *N. Engl. J. Med.*, **325**, 997–1001.
6) Iwasa, S., Fan, J., Shimokama, T., Nagata, M. and Watanabe, T. (1999) Increased immunoreactivity of endothelin-1 and endothelin B receptor in human atherosclerotic lesions. A possible role in atherogenesis. *Atherosclerosis*, **146**, 93–100.
7) Barton, M., Haudenschild, C. C., d'Uscio, L. V., Shaw, S., Munter, K. and Luscher, T. F. (1998) Endothelin ETA receptor blockade restores NO-mediated endothelial function and inhibits atherosclerosis in apolipoprotein E-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 14367–14372.
8) Kahari, V. M., Olsen, D. R., Rhudy, R. W., Carrillo, P., Chen, Y. Q. and Uitto, J. (1992) Transforming growth factor-beta up-regulates elastin gene expression in human skin fibroblasts. Evidence for post-transcriptional modulation. *Lab. Invest.*, **66**, 580–588.
9) Wolfe, B. L., Rich, C. B., Goud, H. D., Terpstra, A. J., Bashir, M., Rosenbloom, J., Sonenshein, G. E. and Foster, J. A. (1993) Insulin-like growth factor-I regulates transcription of the elastin gene. *J. Biol. Chem.*, **268**, 12418–12426.
10) Mauviel, A., Chen, Y. Q., Kahari, V. M., Ledo, I., Wu, M., Rudnicka, L. and Uitto, J. (1993) Human recombinant interleukin-1 beta up-regulates elastin gene expression in dermal fibroblasts. Evidence for transcriptional regulation in vitro and in vivo. *J. Biol. Chem.*, **268**, 6520–6524.
11) Tokimitsu, I., Tajima, S. and Nishikawa, T. (1990) Preferential inhibition of elastin synthesis by epidermal growth factor in chick aortic smooth muscle cells. *Biochem. Biophys. Res. Commun.*, **168**, 850–856.
12) Mecham, R. P., Morris, S. L., Levy, B. D. and Wrenn, D. S. (1984) Glucocorticoids stimulate elastin production in differentiated bovine ligament fibroblasts but do not induce elastin synthesis in undifferentiated cells. *J. Biol. Chem.*, **259**, 12414–12418.
13) Mecham, R. P., Levy, B. D., Morris, S. L., Madaras, J. G. and Wrenn, D. S. (1985) Increased cyclic GMP levels lead to a stimulation of elastin production in

ligament fibroblasts that is reversed by cyclic AMP. *J. Biol. Chem.*, **260**, 3255–3258.
14) Levy, B. I., Michel, J. B., Salzmann, J. L., Azizi, M., Poitevin, P., Safar, M. and Camilleri, J. P. (1988) Effects of chronic inhibition of converting enzyme on mechanical and structural properties of arteries in rat renovascular hypertension. *Circ. Res.*, **63**, 227–239.
15) Ross, R. (1981) George Lyman Duff Memorial Lecture. Atherosclerosis: a problem of the biology of arterial wall cells and their interactions with blood components *Arteriosclerosis*, **1**, 293–311.
16) Oakes, B. W., Batty, A. C., Handley, C. J. and Sandberg, L. B. (1982) The synthesis of elastin, collagen, and glycosaminoglycans by high density primary cultures of neonatal rat aortic smooth muscle. An ultrastructural and biochemical study. *Eur. J. Cell Biol.*, **27**, 34–46.
17) Chomczynski, P. and Sacchi, N., (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159.
18) Tokimitsu, I., Tajima, S., Nishikawa, T., Tajima, M. and Fukasawa, T. (1987) Sequence analysis of elastin cDNA from chick aorta and tissue-specific transcription of the elastin gene in developing chick embryo. *Arch. Biochem. Biophys.*, **256**, 455–461.
19) Cleveland, D. W., Lopata, M. A., MacDonald, R. J., Cowan, N. J., Rutter, W. J. and Kirschner, M. W. (1980) Number and evolutionary conservation of alpha- and beta-tubulin and cytoplasmic beta- and gamma-actin genes using specific cloned cDNA probes. *Cell*, **20**, 95–105.
20) Wu, Y., Rich, C. B., Lincecum, J., Trackman P. C., Kagan, H. M. and Foster, J. A. (1992) Characterization and developmental expression of chick aortic lysyl oxidase. *J. Biol. Chem.*, **267**, 24199–24206.
21) Falanga, V., Katz, M. H., Kirsner, R. and Alvarez, A. F. (1992) The effects of endothelin-1 on human dermal fibroblast growth and synthetic activity. *J. Surg. Res.*, **53**, 515–519.
22) Tokimitsu, I. and Tajima, S. (1994) Inhibition of elastin synthesis by high potassium salt is mediated by Ca2+ influx in cultured smooth muscle cells in vitro: reciprocal effects of K+ on elastin and collagen synthesis. *J. Biochem.* (Tokyo), **115**, 536–539.
23) Hayashi, H., Wachi, H. and Tajima, S. (1995) Presence of elastin-related 45-kDa fragment in culture medium: specific cleavage product of tropoelastin in vascular smooth muscle cell culture. *Biochim. Biophys. Acta*, **1244**, 325–330.
24) Bressan, G. M., Argos, P. and Stanley, K. K. (1987) Repeating structure of chick tropoelastin revealed by complementary DNA cloning. *Biochemistry*, **26**, 1497–1503.
25) Baule, V. J. and Foster, J. A. (1988) Multiple chick tropoelastin mRNAs. *Biochem. Biophys. Res. Commun.*, **154**, 1054–1060.
26) Wachi, H., Seyama, Y., Yamashita, S. and Tajima, S. (1995) Cell cycle-dependent regulation of elastin gene in cultured chick vascular smooth-muscle cells. *Biochem. J.*, **309**, 575–579.
27) Parks, W. C., Kolodziej, M. E. and Pierce, R. A. (1992) Phorbol ester-mediated downregulation of tropoelastin expression is controlled by a posttranscriptional mechanism. *Biochemistry*, **31**, 6639–6645.
28) Tokimitsu, I., Kato, H., Wachi, H. and Tajima, S. (1994) Elastin synthesis is inhibited by angiotensin II but not by platelet-derived growth factor in arterial smooth muscle cells. *Biochim. Biophys. Acta*, **1207**, 68–73.
29) Hayashi, A., Suzuki, T., Wachi, H., Tajima, S., Nishikawa, T., Murad, S. and Pinnell, S. R. (1994) Minoxidil stimulates elastin expression in aortic smooth muscle cells. *Arch. Biochem. Biophys.*, **315**, 137–141.
30) Hayashi, A., Suzuki, T. and Tajima, S. (1995) Modulations of elastin expression and cell proliferation by retinoids in cultured vascular smooth muscle cells. *J. Biochem.* (Tokyo), **117**, 132–136.
31) Wachi, H., Seyama, Y. and Tajima, S. (1995) Modulation of elastin expression by heparin is dependent on the growth condition of vascular smooth muscle cells: up-regulation of elastin expression by heparin in the proliferating cells is mediated by the inhibition of protein kinase C activity. *J. Biochem.* (Tokyo), **118**, 582–586.
32) Zamora, M. A., Dempsey, E. C., Walchak, S. J. and Stelzner, T. J. (1993) BQ123, an ETA receptor antagonist, inhibits endothelin-1-mediated proliferation of human pulmonary artery smooth muscle cells. *Am. J. Respir Cell Mol. Biol.*, **9**, 429–433.
33) Takuwa, Y., Kasuya, Y., Takuwa, N., Kudo, M., Yanagisawa, M., Goto, K., Masaki, T. and Yamashita, K. (1990) Endothelin receptor is coupled to phospholipase C via a pertussis toxin-insensitive guanine nucleotide-binding regulatory protein in vascular smooth muscle cells. *J. Clin. Invest.*, **85**, 653–658.
34) Smith-Mungo, L. I. and Kagan, H. M. (1988) Lysyl oxidase: properties, regulation and multiple functions in biology. *Matrix Biol.*, **16**, 387–398.
35) Gacheru, S. N., Thomas, K. M., Murray, S. A., Csiszar, K., Smith-Mungo, L. I. and Kagan, H. M. (1997) Transcriptional and post-transcriptional control of lysyl oxidase expression in vascular smooth muscle cells: effects of TGF-beta 1 and serum deprivation. *J. Cell Biochem.*, **65**, 395–407.
36) Roy, R., Polgar, P., Wang, Y., Goldstein, R. H.,

Taylor, L. and Kagan, H. M. (1996) Regulation of lysyl oxidase and cyclooxygenase expression in human lung fibroblasts: interactions among TGF-β, IL-1 beta, and prostaglandin E. *J. Cell Biochem.*, **62**, 411–417.

37) Green, R. S., Lieb, M. E., Weintraub, A. S., Gacheru, S. N., Rosenfield, C. L., Shah, S., Kagan, H. M. and Taubman, M. B. (1995) Identification of lysyl oxidase and other platelet-derived growth factor-inducible genes in vascular smooth muscle cells by differential screening. *Lab. Invest.*, **73**, 476–482.
38) Ravid, K., Smith-Mungo, L. I., Zhao, Z., Thomas, K. M. and Kagan, H. M. (1999) Upregulation of lysyl oxidase in vascular smooth muscle cells by cAMP: role for adenosine receptor activation. *J. Cell Biochem.*, **75**, 177–185.
39) Dubey, R. K., Gillespie, D. G., Osaka, K., Suzuki, F. and Jackson, E. K. (1996) Adenosine inhibits growth of rat aortic smooth muscle cells. Possible role of A2b receptor. *Hypertension*, **27**, 786–793.
40) Kramsch, D. M. and Hollander, W. (1973) The interaction of serum and arterial lipoproteins with elastin of the arterial intima and its role in the lipid accumulation in atherosclerotic plaques. *J. Clin. Invest.*, **52**, 236–247.
41) Saulnier, J. M., Hauck, M., Fulop, T., Jr. and Wallach, J. M. (1991) Human aortic elastin from normal individuals and atherosclerotic patients: lipid and cation contents; susceptibility to elastolysis. *Clin. Chim. Acta*, **200**, 129–136.