Vol. 209, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 6, 1995 Pages 175-181

SUPPRESSION OF ELASTIN GENE EXPRESSION IN DERMAL FIBROBLASTS BY PROTEIN PHOSPHATASE INHIBITOR OKADAIC ACID

Jukka Westermarck[2,3], Elina Ilvonen[1,3], Jouni Uitto[4] and Veli-Matti Kähäri[1,3,*]

Departments of [1] Dermatology and [2] Medical Biochemistry, and [3] MediCity Research Laboratory, University of Turku, FIN-20520 Turku, Finland

[4] Department of Dermatology, Thomas Jefferson University, Philadelphia, PA 19107

Received February 22, 1995

Treatment of human skin fibroblasts with okadaic acid, an inhibitor of serine/threonine specific protein phosphatases 1 and 2A, resulted in a marked reduction in elastin mRNA levels, with maximal inhibition of 80%. The inhibitory effect of okadaic acid on elastin mRNA levels was efficiently prevented by retinoic acid and cycloheximide and was further enhanced by phorbol ester treatment. Upregulation of elastin mRNA levels by transforming growth factor-β2 was abrogated by simultaneous treatment of cells with okadaic acid. Okadaic acid had no effect on the expression of human elastin promoter/CAT chimeric gene in fibroblasts cultured from skin of transgenic mice. These results provide evidence that protein phosphatases 1 and 2A play an important role as positive regulators of elastin gene expression in dermal fibroblasts.
© 1995 Academic Press, Inc.

Elastic fibers are extracellular matrix components primarily responsible for the resilience and elasticity of tissues, such as skin, lungs and blood vessels. The major component of elastic fibers is elastin, a highly insoluble protein which is associated with the microfibrillar component containing fibrillin (1-3). Several growth factors and

*To whom correspondence should be addressed at Department of Dermatology, University of Turku, Kiinamyllynkatu 4-8, FIN-20520 Turku, Finland. Fax: 358-21-6337000.

Abbreviations used: OA, okadaic acid; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; TGF-β, transforming growth factor-β; TPA, 12-O-tetradecanoyl-13-phorbol acetate.

0006-291X/95 $5.00
Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

cytokines regulate expression of the elastin gene. For example, transforming growth factor-β (TGF-β) is a potent stimulator of elastin gene expression in smooth muscle cells and dermal fibroblasts (4-6). In addition, interleukin-1β and insulin like growth factor-1 enhance elastin gene expression at the transcriptional level (7,8). On the other hand, expression of elastin gene is potently down-regulated by tumor necrosis factor-α and phorbol ester TPA at the transcriptional level (9) and by interferon-γ, dexamethasone and vitamin D_3 at least in part at the post-transcriptional level (9-11). Cloning of the entire human elastin gene, and analysis of 2.2 kb of the 5'-flanking region have revealed several positive and negative regulatory regions, as well as several putative regulatory *cis*-elements (1,2,12).

Both DNA binding and *trans*-activating capacity of transcription factors is regulated by phosphorylation as a result of balance between the activity of protein kinases and protein phosphatases (13). Okadaic acid (OA) is a novel, non-phorbol ester type tumor promoter, which is an inhibitor of cellular phosphoserine and phosphothreonine specific protein phosphatases 1 and 2A (PP1 and PP2A) (14,15). We and others have shown that inhibition of PP1 and PP2A by OA results in enhanced matrix degradation capacity by inducing collagenase and stromelysin-1 gene expression in various types of cells (16,17). In this study we show that inhibition of PP1 and PP2A by OA markedly reduces elastin mRNA levels in human skin fibroblasts in culture. These results provide evidence that PP1 and PP2A play an important role as positive regulators of elastin gene expression in dermal fibroblasts.

MATERIALS AND METHODS

Cell cultures. Human skin fibroblast cultures established from a voluntary healthy male donor (age 28) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). The cells were incubated with OA (Sigma Chemical Co., St. Louis, MO, or LC Laboratories, Woburn, MA) in concentrations indicated for 24 h. In some experiments, cycloheximide (10 μg/ml), retinoic acid (1 μM) or 12-O-tetradecanoyl-13-phorbol acetate (TPA) (60 ng/ml) (all from Sigma), or TGF-β2 (a gift from Dr. David Olsen, Celtrix Laboratories, Santa Clara, CA), were added to the culture media 1 hr prior to addition of OA. To estimate the viability of cells after 24 h incubation with OA, cells were washed with phosphate buffered saline (PBS) and stained with 0.4% trypan blue in PBS for 10 min. Cells were then washed with PBS, fixed with 10% formaldehyde and the number of stained cells was counted.

RNA analysis. Total cellular RNA was isolated from cells using guanidine thiocyanate/cesium chloride method (18). Aliquots of total RNA (10 or 15 μg) were fractionated on 0.8% agarose gels containing 2.2 M formaldehyde and transferred to Zeta Probe filter (BioRad, Richmond, CA). The RNA samples were stained with ethidium bromide and visualized by UV-light to check the loading of the samples. The filters were prehybridized for 2 h and subsequently hybridized for 20 h with DNA probes labeled with [α-^{32}P]dCTP using random priming. The filters were then washed in the final stringency of 0.1 x SSC/0.1% SDS at 60°C (19). The [^{32}P]cDNA-mRNA hybrids were visualized by autoradiography, and quantitated by scanning densitometry of the autoradiographs. The following probes were used for

hybridizations: for elastin, a 3.2-kb human elastin cDNA (cHDE-1) (20); for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a 1.3-kb rat cDNA (21).

Determination of elastin promoter activity. To examine the effect of OA on elastin promoter activity, we used fibroblasts cultured from the skin of transgenic mice harboring in their genome a 5.2-kb human elastin promoter fragment linked to CAT reporter gene (22). The cells were treated with varying concentrations of OA for 36 h. The cells were harvested and CAT activity was measured as an index of promoter activity (23). Acetylated and non-acetylated forms of [^{14}C]chloramphenicol were separated on thin layer chromatography, visualized and quantified with Model GS-250 Molecular Imager System and Phosphor Analyst Software (BioRad, Hercules, CA).

RESULTS AND DISCUSSION

To examine the effect of OA on elastin gene expression, confluent cultures of normal human skin fibroblasts maintained in DMEM supplemented with 10% FCS were treated with various concentrations of OA for 24 h. Thereafter, elastin mRNA abundance was estimated by Northern blot hybridization. The results show that OA reduced elastin mRNA levels in a dose-dependent manner, the maximal inhibition detected with concentration 30 ng/ml (Fig. 1A). Quantitation of elastin mRNA, after correction for GAPDH mRNA levels, revealed that the maximal inhibition noted with OA concentration 30 ng/ml was 70% (Fig. 1B). Treatment of dermal fibroblasts with OA in all concentrations had no effect on the viability of cells, as estimated by trypan blue exclusion (not shown).

In the next experiment we examined whether the OA-elicited reduction in elastin mRNA levels is dependent on on-going protein synthesis. Confluent human skin fibroblast cultures were treated with OA (20 ng/ml) alone or in combination with cycloheximide (10 μg/ml) in DMEM supplemented with 1% FCS. Under the low serum conditions, OA suppressed elastin mRNA levels by 80%. The OA-elicited suppression of elastin mRNA abundance was effectively prevented by cycloheximide (Fig. 2A,B),

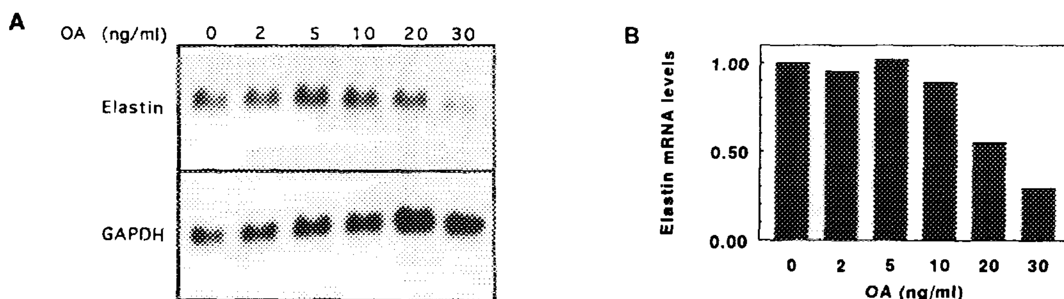

Figure 1. Dose-dependent reduction of elastin mRNA levels by OA in human skin fibroblasts. (A) Confluent human skin fibroblast cultures were incubated for 24 h in DMEM containing 10% FCS with various concentrations of OA as indicated. Total RNA (15 μg/lane) was analyzed by Northern blot hybridizations with the cDNA probes for elastin and GAPDH, as indicated. (B) Elastin mRNA levels were quantitated by densitometric scanning of the X-ray film in (A), corrected for GAPDH mRNA levels in the same samples, and expressed relative to the levels in the untreated control cultures (1.00).

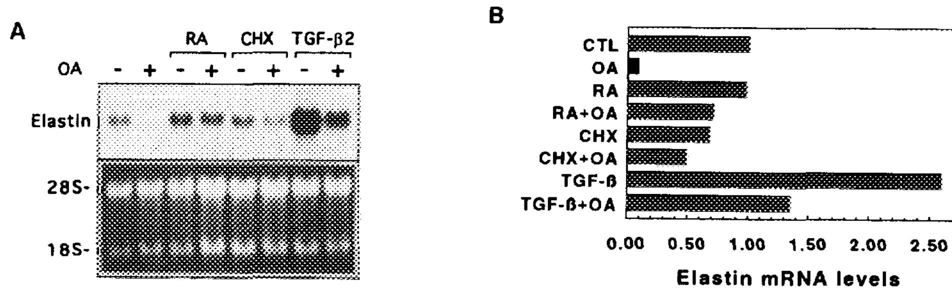

Figure 2. Effect of OA on elastin mRNA levels in combination with retinoic acid, cycloheximide, and TGF-β. (A) Confluent human skin fibroblast cultures were incubated for 24 h in DMEM containing 1% FCS with okadaic acid (OA) (20 ng/ml) either alone, or in combination with retinoic acid (RA; 1 μM), cycloheximide (CHX; 10 μg/ml) or transforming growth factor-β2 (TGF-β2; 5 ng/ml), all added 1 h prior to OA. Total RNA (15 μg/lane) was analyzed by Northern blot hybridization with the cDNA probe for elastin. The amount of rRNA in the same samples was estimated by ethidium bromide staining (lower panel). (B) Elastin mRNA levels were quantitated by densitometric scanning of the X-ray film in (A), corrected for the levels of rRNA in the same samples, and expressed relative to the levels in the untreated control cultures (CTL; 1.00).

indicating that the inhibitory effect of OA on elastin gene expression is, at least in part, dependent on continuous protein synthesis.

In the same experiment, skin fibroblasts were simultaneously treated with OA and retinoic acid (1 μM). In these cells, retinoic acid alone had no effect on elastin mRNA levels (Fig. 2A,B). However, exposure of cells to retinoic acid potently prevented the inhibitory effect of OA on elastin mRNA levels (Fig. 2A,B).

TGF-β is a potent stimulator of elastin gene expression in dermal fibroblasts and smooth muscle cells (4-6). In this context we also wanted to examine the effect of OA

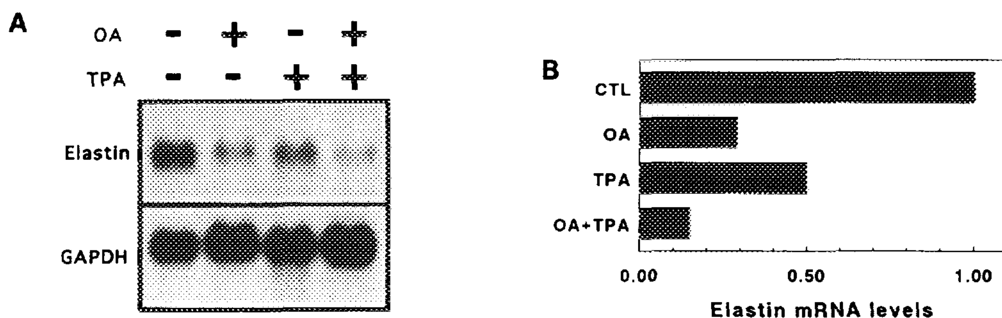

Figure 3. OA augments down-regulation of elastin mRNA levels by TPA. (A) Confluent human skin fibroblast cultures were incubated for 24 h in DMEM containing 1% FCS with (+) or without (-) OA (10 ng/ml) added 1 h prior to addition of TPA (60 ng/ml). Total RNA (10 μg/lane) was analyzed by Northern blot hybridizations with the cDNA probes for elastin and GAPDH. (B) Elastin mRNA levels were quantitated by densitometric scanning of the X-ray film in (A), corrected for GAPDH mRNA levels in the same samples, and expressed relative to the levels in the untreated control cultures (CTL; 1.00).

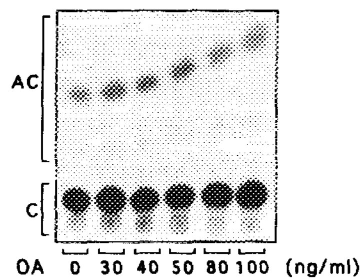

Figure 4. Elastin promoter activity is not altered by OA. Confluent cultures of dermal fibroblasts from transgenic mice, carrying a 5.2-kb human elastin promoter fragment linked to CAT reporter gene, were treated for 36 h in DMEM containing 1% FCS with various concentrations of OA, as indicated. Cells were then harvested and equal amounts of protein were measured for CAT activity as an index of promoter activity. A representative CAT assay is shown. AC and C represent acetylated and non-acetylated forms of [^{14}C]chloramphenicol, respectively.

on elastin gene expression in combination with TGF-β2. Treatment of cells with TGF-β2 (5 ng/ml) potently enhanced the abundance of elastin gene transcripts (Fig. 2A,B). Interestingly, treatment of cells with OA abrogated the upregulation of elastin mRNA levels by TGF-β2 (Fig. 2A,B).

We have previously shown that elastin gene expression by dermal fibroblasts is inhibited by phorbol ester TPA (9). In the next experiment, TPA alone reduced elastin mRNA levels by 50%, and OA by 70% (Fig. 3A,B). Simultaneous treatment of cells with TPA and OA resulted in a further inhibition of elastin mRNA levels, by a total of 85%, as compared to the untreated control cultures (Fig. 3A,B). These results indicate that OA and TPA act synergistically on elastin gene expression.

Given the potent inhibitory effect of OA on elastin mRNA abundance, we studied the effect of OA on elastin promoter activity in fibroblasts cultured from the skin of transgenic mice harboring in their genome a 5.2-kb human elastin promoter fragment linked to CAT reporter gene (22). Expression of this chimeric gene in these cells has been shown to be susceptible to modulation by interleukin-1β (7). Treatment of these cells with varying concentrations of OA for a period of 36 h had no marked effect on the elastin promoter driven CAT activity in these cells, indicating that the inhibitory effect of OA on elastin gene does not take place at transcriptional level (Fig. 4).

The results of the present study provide evidence that cellular PP1 and PP2A play an important role as positive regulators of elastin gene expression, and that specific inhibition of their activity by OA results in potent suppression of elastin gene expression in human skin fibroblasts. These observations also suggest that the ability of retinoic acid to restore down-regulated elastin gene expression may be useful in disorders characterized by loss of elastic fibers in connective tissue, such as anetoderma (24).

Increased accumulation of elastic fibers as a result of enhanced elastin and fibrillin gene expression has been reported in photodamaged skin (25). This may represent a

direct effect of UV light on dermal fibroblasts, or may be indirectly mediated by cytokines derived from epidermal keratinocytes or inflammatory cells. The results presented in this study suggest that selective inhibition of PP1 and PP2A may provide a novel way of suppressing elastin gene expression in clinical conditions characterized by enhanced expression of elastin, including photodamage of the skin.

ACKNOWLEDGMENTS

We thank Ms. Marita Potila for skillful technical assistance. This study was financially supported by grants from Paulo Foundation, Turku University Foundation, the Cancer Research Foundation of the Southwestern Finland, and Sigrid Jusélius Foundation (to V.-M.K.), and by a USPHS/NIH grant RO1-AR28450 (to J.U.).

REFERENCES

1. Uitto, J., Christiano, A.M., Kähäri, V-M., Bashir, M.M., and Rosenbloom, J. (1991) Biochem. Soc. Transact. 19, 824-829.
2. Rosenbloom, J., Bashir, M., Kähäri, V-M., and Uitto, J. (1991) Crit. Rev. Eukaryot. Gene Expr. 1, 145-156.
3. Rosenbloom, J., Abrams, W.R. , and Mecham, R. (1994) FASEB J. 7, 1208-1218.
4. Liu, J., and Davidson, J. (1988) Biochem. Biophys. Res. Commun. 154, 895-901.
5. Kähäri, V-M., Olsen, D.R., Rhudy, R.W., Carrillo, P., Chen, Y.Q., and Uitto, J. (1992) Lab. Invest. 66, 580-588.
6. Katchman, S.D., Hsu-Wong, S., Ledo, I., Wu, M., and Uitto, J. (1994) Biochem. Biophys. Res. Commun. 203, 485-490.
7. Mauviel, A., Chen, Y.Q., Kähäri, V-M., Ledo, I., Wu, M., Rudnicka, L., and Uitto, J. (1993) J. Biol. Chem. 268, 6520-6524.
8. Wolfe, B.L., Rich, C.B., Goud, H.D., Terpstra, A.J., Bashir, M., Rosenbloom, J, Sonenshein, G.E., and Foster, J.A. (1993) J. Biol. Chem. 268, 12418-12426.
9. Kähäri, V-M., Chen, Y.Q., Bashir, M.M., Rosenbloom, J., and Uitto, J. (1992) J. Biol. Chem. 267, 26134-26141.
10. Kähäri, V-M. (1994) Biochem. Biohys. Res. Commun. 201, 1189-1196.
11. Pierce, R.A., Kolodziej, M.E., and Parks, W.C. (1992) J. Biol. Chem. 267, 11593-11599.
12. Kähäri, V-M., Fazio, M.J., Chen, Y.Q., Bashir, M.M., Rosenbloom, J., and Uitto, J. (1990) J. Biol. Chem. 265, 9485-9490.
13. Hunter, T., and Karin, M. (1992) Cell 70, 375-387.
14. Schönthal, A. (1992) New Biol. 4, 16-21.
15. Shenolikar, S. (1994) Ann. Rev. Cell Biol. 10, 55-86.
16. Kim, S-J., Lafyatis, R., Kim, K.Y., Angel, P., Fujiki, H., Karin, M. Sporn, M.B., and Roberts, A.B. (1990) Cell Regulation 1, 269-278.
17. Westermarck, J., Lohi, J., Keski-Oja, J., and Kähäri, V-M. (1994) Cell Growth Differ. 5, 1205-1213.
18. Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1983) Biochemistry 18, 5924-5929.
19. Thomas, P.S. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 5201-5205.
20. Fazio, M.J., Olsen, D.R., Kauh, E.A., Baldwin, C.T., Indik, Z., Ornstein-Goldstein, N., Yeh, H., Rosenbloom, J., and Uitto, J. (1988) J. Invest. Dermatol. 91, 458-464.

21. Fort, P., Marty, L., Piechaczyk, M., El Sabrouty S., Dani, C., Jeanteur, P., and Blanchard, J.M. (1985) Nucleic Acids Res. 13, 1431-1442.
22. Hsu-Wong, S., Katchman S.D., Ledo, I., Wu, M., Khillan, J., Bashir, M.M., Rosenbloom, J., and Uitto, J. (1994) J. Biol. Chem. 269, 18072-18075.
23. Gorman, C.M., Moffat, L.F., and Howard, B.H. (1982) Mol. Cell. Biol. 2, 1044-1051.
24. Oikarinen, A.I., Palatsi, R., Adomian, B.E., Clark, J.G., and Uitto, J. (1984) J. Am. Acad. Dermatol. 11, 64-72.
25. Bernstein, E.F., Chen, Y.Q., Tamai, K., Shepley, K.J., Resnik, K.S., Zhang, H., Tuan, R., Mauviel, A., and Uitto, J. (1994) J. Invest. Dermatol. 103, 182-186.