The protein phosphatase inhibitor okadaic acid suppresses type I collagen gene expression in cultured fibroblasts at the transcriptional level

Jukka WESTERMARCK,*† Elina ILVONEN,†‡ and Veli-Matti KÄHÄRI†‡§

*Department of Medical Biochemistry, †MediCity Research Laboratory and ‡Department of Dermatology, University of Turku, FIN-20520 Turku, Finland

Type I collagen is the most abundant component of the extracellular matrix of human connective tissues. We have examined the effect of okadaic acid (OA), an inhibitor of phosphoserine- and phosphothreonine-specific protein phosphatases 1 and 2A, on type I collagen gene expression by fibroblasts in culture. Treatment of human skin fibroblasts with OA potentially reduced type I and type III collagen mRNA levels, maximally by over 90%. The inhibitory effect of OA on type I and III collagen mRNA abundance was not prevented by cycloheximide, and was not affected by simultaneous treatment with dexamethasone or retinoic acid. OA also abrogated the enhancing effect of transforming growth factor- β (TGF- β) on type I and III collagen

mRNA levels. Treatment of transiently transfected NIH-3T3 fibroblasts with OA suppressed the activity of a 3.5 kb human pro α 2(I) collagen promoter/chloramphenicol acetyltransferase construct maximally, by 70%. In addition, OA treatment of NIH-3T3 cells abrogated enhancement of pro α 2(I) collagen promoter activity by TGF- β . These results indicate that protein phosphatases 1 and 2A have an important role as positive regulators of type I and III collagen gene expression. The results also suggest that selective inhibition of activity of protein phosphatases 1 and 2A may offer a novel approach for preventing excessive collagen accumulation in fibrotic disorders.

INTRODUCTION

Fibrillar collagen of type I is an abundant component of the extracellular matrix of different connective tissues, including skin (see [1,2]). Type I collagen molecules are heterotrimers which consist of two α 1(I) chains and one α 2(I) chain. Expression of pro α 1(I) and pro α 2(I) collagen genes is co-ordinately regulated at both transcriptional and post-transcriptional level, resulting in synthesis of pro α 1(I) and pro α 2(I) collagen chains in a 2:1 ratio [1,2].

Formation of extracellular matrix is regulated by a number of polypeptide growth factors, many of which are secreted by mononuclear inflammatory cells [3]. The most potent stimulator of type I collagen gene expression is transforming growth factor- β (TGF- β), which enhances expression of type I collagen genes both at transcriptional and post-transcriptional level [4–6]. Type I collagen gene expression is potentially inhibited at promoter level by tumor necrosis factor- α (TNF- α), which also abrogates the transcriptional activation of type I collagen genes by TGF- β [5,7]. On the other hand, interferon- γ (IFN- γ) and glucocorticoids potentially suppress expression of type I collagen genes at post-transcriptional level [5,8,9].

Accumulation of type I collagen is strictly regulated in physiological situations such as embryonal development of tissues and wound repair [10–12]. On the other hand, uncontrolled accumulation of type I collagen results in tissue fibrosis in disorders such as scleroderma, keloids and pulmonary fibrosis (see [2,13]). In these fibrotic conditions, either TGF- β 1 or TGF- β 2 has been implicated in the pathogenetic chain, resulting in excessive collagen deposition [14–18].

Several regulatory *cis*-elements, controlling the tissue-specific expression of type I collagen, have been identified in the 5'-flanking regions of both pro α 1(I) and pro α 2(I) collagen genes [19–24]. Recently, the role of phosphorylation in the regulation of DNA binding and *trans*-activating capacity of transcription factors has been emphasized [25,26]. The phosphorylation state of *trans*-acting factors is modulated by activity of protein kinases and protein phosphatases. Okadaic acid (OA) is a novel, non-phorbol-ester-type tumour promoter, which is a specific inhibitor of cellular phosphoserine- and phosphothreonine-specific protein phosphatases 1 and 2A (PP1 and PP2A) (see [27]). We and others have shown that OA enhances the matrix-degradative capacity of several types of cells by enhancing expression of interstitial collagenase (matrix metalloproteinase-1; MMP-1) and stromelysin-1 (matrix metalloproteinase-3; MMP-3) [28,29]. In the present study we show that OA potentially suppresses type I collagen gene expression at the mRNA and promoter level, and can abrogate the enhancing effect of TGF- β on type I collagen gene expression. The results indicate that selective inhibition of PP1 and PP2A activity may serve as a novel approach in preventing collagen accumulation in fibrotic disorders.

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MATERIALS AND METHODS

Cell culture

Normal human skin fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%

Abbreviations used: OA, okadaic acid; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; TGF- β , transforming growth factor- β ; TNF- α , tumour necrosis factor- α ; MMP, matrix metalloproteinase; CAT, chloramphenicol acetyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TIMP-1, tissue inhibitor of metalloproteinases-1; IFN- γ , interferon- γ ; DMEM, Dulbecco's modified Eagle's medium; (F)CS, (fetal-) calf serum.

§ To whom correspondence should be sent.

fetal-calf serum (FCS), 2 μ M glutamine, 100 i.u./ml penicillin-G and 100 μ g/ml of streptomycin. Mouse NIH-3T3 cells (from the American Type Culture Collection, Rockville, MD, U.S.A.) were cultured in the same medium supplemented with 10% calf serum (CS). For RNA analysis, skin fibroblasts were maintained in DMEM supplemented with 1 or 10% FCS for 18 h. Thereafter, OA in the free-acid form (from Sigma Chemical Co., St. Louis, MO, U.S.A.), or as sodium salt (from LC Laboratories, Woburn, MA, U.S.A.) was added in the concentrations indicated, and the incubations were continued for 24 h. In some experiments, cycloheximide (10 μ g/ml), dexamethasone (1 μ M), retinoic acid (1 μ M) (all from Sigma) or TGF- β 2 (kindly provided by Dr. David R. Olsen, Celtrix Co., Santa Clara, CA, U.S.A.), was added to the culture medium 1 h prior to addition of OA. To estimate the viability of cells after 24 h incubation with OA, cells were washed with PBS and stained with 0.4% Trypan Blue in PBS for 10 min. Cells were then washed again with PBS, fixed with 10% formaldehyde, and the number of stained cells was counted.

RNA Analysis

Total cellular RNA was isolated from cell cultures by using the guanidine thiocyanate/cesium chloride method [30]. Aliquots of total RNA (10 or 15 μ g) were fractionated on 0.8% agarose gels containing 2.2 M formaldehyde and transferred to Zeta probe filter (Bio-Rad, Richmond, CA, U.S.A.) by vacuum transfer (VacuGene XL; LKB, Bromma, Sweden) and immobilized by heating at 80 °C for 30 min. The filters were prehybridized for 2 h and subsequently hybridized for 20 h with DNA probes labelled with [α - 32 P]dCTP using random priming. The filters were then washed, the final stringency of washes being $0.1 \times \text{SSC}/0.1\%$ SDS at 60 °C [31] ($1 \times \text{SSC}$ is 0.15 M NaCl/15 mM sodium citrate, pH 7.0). The [32 P]cDNA-mRNA hybrids were revealed by autoradiography, and the mRNA levels were quantified by scanning densitometry of the autoradiographs using MCID software (Imaging Research Inc., St. Catharines, Ontario, Canada). The following probes were used for hybridizations: for pro α 1(I) collagen, a 0.7 kb human cDNA [32]; for pro α 2(I) collagen, a 1.2 kb human cDNA [33]; for pro α 1(III) collagen, a 0.7 kb human cDNA [34]; for collagenase, a 2.0 kb human cDNA [35]; for stromelysin-1, a 1.5 kb human cDNA [36]; for tissue inhibitor of metalloproteinases-1 (TIMP-1), a 0.7 kb human cDNA [37]; for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a 1.3 kb rat cDNA [38].

Transient transfections

Confluent mouse NIH-3T3 cell cultures were transiently transfected with 10 μ g of pro α 2(I) collagen promoter/chloramphenicol acetyltransferase (CAT) construct pMS3.5/CAT (kindly provided by Dr. Francesco Ramirez, Mount Sinai School of Medicine, New York, NY, U.S.A.). This construct contains 3.5 kb of the 5'-flanking region of human pro α 2(I) collagen gene linked to the CAT reporter gene [20]. Transfections were performed by the calcium phosphate/DNA co-precipitation method, followed by a 2 min glycerol shock [39]. At 4 h after the glycerol shock, various concentrations of OA were added and the incubations were continued for 36 h. Cells were then harvested and CAT activity was measured as an index of promoter activity, as described previously [40]. Acetylated and non-acetylated forms of [14 C]chloramphenicol were separated by TLC, revealed by autoradiography and quantified by using a Model GS-250 Molecular Imager System and Phosphor Analyst Software (Bio-Rad, Hercules, CA, U.S.A.). Transfection efficiency was monitored by co-transfecting the promoter/CAT construct with

5 μ g of Rous sarcoma virus/ β -galactosidase construct and correcting the CAT activities for β -galactosidase activity.

In experiments involving TGF- β , cells were transfected on 60 mm-diameter dishes as described above. At 4 h after the glycerol shock, cells were treated with trypsin, pooled, then seeded on 40 mm-diameter dishes. After 12 h, TGF- β 1 or TGF- β 2 (5 ng/ml) was added alone or in combination with OA (80 ng/ml), and the incubations were continued for 30 h. The cells were harvested, and aliquots corresponding to equal amounts of protein were used to measure CAT activity.

RESULTS

Dose-dependent reduction of type I and III collagen mRNA levels by OA

Initially, normal human skin fibroblasts in DMEM containing 10% FCS were exposed to various concentrations of OA for a period of 24 h, and mRNA levels were determined by Northern-blot hybridizations. Treatment of dermal fibroblasts with OA resulted in a detectable reduction in both pro α 1(I) and pro α 2(I) collagen mRNA abundance with concentrations 20 and 30 ng/ml (Figure 1). The mRNA levels for pro α 1(I) and pro α 2(I) collagen mRNA were maximally reduced by 83 and 90% respectively with OA concentration 30 ng/ml. The levels for pro α 1(III) collagen mRNA were reduced to a lesser extent, maximally by 48% with OA concentration 30 ng/ml (Figure 1). Treatment of dermal fibroblasts with OA in all concentrations had no effect on the viability of cells, as estimated by Trypan Blue exclusion (results not shown).

OA-elicited reduction in type I and III collagen mRNA levels is not dependent on protein synthesis

In the next experiment, human skin fibroblasts were treated with OA in DMEM containing 1% FCS. Under these low-serum conditions, OA (20 ng/ml) reduced pro α 1(I) and pro α 2(I) collagen mRNA levels by 92% and 93% respectively and pro α 1(III) collagen mRNA levels by 97% as compared with the levels in untreated control cultures (Figure 2). To examine whether OA-elicited reduction in type I or III collagen mRNA

Figure 1 Dose-dependent reduction of type I and III collagen mRNA levels by OA in human skin fibroblasts

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 μ g/lane) was analysed by Northern-blot hybridizations with cDNA probes for pro α 1(I), pro α 2(I) and pro α 1(III) collagen and GAPDH.

Figure 2 OA inhibits TGF- β -elicited enhancement of type I and III collagen mRNA levels

Confluent human skin fibroblast cultures were incubated for 24 h in DMEM containing 1% FCS, with OA (20 ng/ml), either alone or in combination with retinoic acid (RA, 1 μ M), cycloheximide (CHX, 10 μ g/ml) or TGF- β 2 (5 ng/ml), all added 1 h prior to OA. Aliquots of total RNA (15 μ g/lane) were analysed by Northern-blot hybridizations with the cDNA probes indicated.

levels is dependent on protein synthesis, fibroblasts were exposed to cycloheximide in combination with OA. Cycloheximide (10 μ g/ml) alone had no effect on type I or III collagen mRNA levels, nor was it able to prevent suppression of type I and III collagen mRNA levels by OA, indicating that OA-elicited down-regulation of type I and III collagen gene expression does not require new protein synthesis (Figure 2).

In this experiment, treatment of cells with retinoic acid (1 μ M) did not alter pro α 1(I) or pro α 2(I) collagen mRNA levels, and had no effect on OA-elicited inhibition of type I collagen mRNA levels (Figure 2). In contrast, type III collagen mRNA levels were slightly (1.6-fold) increased in cells treated with retinoic acid. However, OA-elicited reduction in type III collagen mRNA abundance was not altered by retinoic acid treatment of dermal fibroblasts (Figure 2).

OA inhibits TGF- β -elicited enhancement of type I and III collagen mRNA levels

Both TGF- β 1 and 2 are potent stimulators of type I collagen gene expression, and have been implicated in the pathogenesis of tissue fibrosis [4–6,14–18,41]. Therefore we also wanted to examine whether OA is able to abrogate the TGF- β -elicited activation of type I collagen gene expression at the mRNA level. Human skin fibroblasts were exposed to TGF- β 2 (5 ng/ml) either alone or in combination with OA (20 ng/ml). As expected, TGF- β 2 enhanced pro α 1(I) and pro α 2(I) as well as pro α 1(III) collagen mRNA levels in dermal fibroblasts up to 2.8-fold (Figure 2). Interestingly, treatment of cells with OA in combination with TGF- β 2 prevented up-regulation of both type I and III collagen mRNA levels by TGF- β 2, resulting in reduction in these mRNA levels below the levels noted in the untreated control cells (Figure 2).

Effect of OA on type I collagen mRNA levels in combination with dexamethasone

Glucocorticoids potently suppress type I collagen gene expression at the post-transcriptional level [9]. We also wanted to test the effect of dexamethasone on type I collagen mRNA levels in combination with OA. In this experiment OA (20 ng/ml) reduced

Figure 3 Effect of dexamethasone on OA-elicited reduction in type I collagen, and induction of collagenase and stromelysin-1 mRNA levels

Confluent human skin fibroblasts were incubated for 24 h in DMEM containing 10% FCS with (+) or without (–) OA (20 ng/ml) added 1 h after dexamethasone (DX, 1 μ M) as indicated (+). (a) Total RNA (15 μ g/lane) was analysed by Northern-blot hybridizations with cDNA probes specific for pro α 1(I) and pro α 2(I) collagen and GAPDH. (b) The same filter was rehybridized with cDNA probes for collagenase, stromelysin-1 and TIMP-1.

pro α 1(I) and pro α 2(I) collagen mRNA levels by 78 and 88% respectively (Figure 3a). Incubation of fibroblasts with dexamethasone (1 μ M) alone resulted in reduction of pro α 1(I) and pro α 2(I) collagen mRNA levels by 55 and 35% respectively as compared with the untreated control cultures (Figure 3a). Treatment of cells with dexamethasone together with OA had no marked effect on the inhibitory effect of OA on type I collagen mRNA levels, indicating that the inhibitory effects of OA and dexamethasone on type I collagen gene expression are not synergistic (Figure 3a).

We have previously demonstrated that glucocorticoid treatment potentially abolishes induction of collagenase gene expression by OA in HT-1080 cells [29]. Hybridization of the same filter with collagenase and stromelysin-1 cDNA probes showed that mRNA levels for both these genes were induced 67- and 88-fold respectively, and that induction of both genes by OA was potentially inhibited by dexamethasone (Figure 3b). In contrast, the mRNA levels for TIMP-1, the natural inhibitor of collagenase and stromelysin-1 activity, were not altered by either OA or dexamethasone (Figure 3b).

Suppression of pro α 2(I) collagen promoter activity by OA

In order to examine whether OA-elicited suppression of type I collagen gene expression takes place at the promoter level, mouse NIH-3T3 fibroblasts were transiently transfected with a 3.5 kb human pro α 2(I) collagen promoter/CAT construct. These cells are readily transfectable and express type I collagen mRNA [4,5]. In DMEM supplemented with 5% CS, treatment of NIH-3T3 cells with OA for 36 h resulted in a dose-dependent reduction in pro α 2(I) collagen promoter activity (Figure 4). The maximal suppression of CAT activity, by 70%, was noted with OA concentration 80 ng/ml (Figure 4). These results show that the inhibitory effect of OA on type I collagen gene expression takes place at the transcriptional level. These findings are also in accordance with previous observations indicating differential sensitivity to OA depending on cell type [28].

OA inhibits TGF- β -elicited enhancement of pro α 2(I) collagen promoter activity

Since the results presented above indicate that OA inhibits activity of the pro α 2(I) collagen promoter in transiently transfected NIH-3T3 fibroblasts, we also wanted to test whether OA

Figure 4 Dose-dependent suppression of pro α 2(I) collagen promoter activity by OA

Confluent NIH-3T3 cell cultures were transfected with the 3.5 kb human pro α 2(I) collagen promoter/CAT construct pMS3.5/CAT (5 μ g) and treated with various concentrations of OA, as indicated, in DMEM containing 5% CS for 36 h. Cells were then harvested and CAT activity was measured. The migration positions of acetylated (AC) and non-acetylated (C) [14 C]chloramphenicol are shown on the left. A representative CAT assay is shown.

Figure 5 OA inhibits up-regulation of pro α 2(I) collagen promoter activity by TGF- β

Confluent NIH-3T3 cell cultures were transfected with 3.5 kb human pro α 2(I) collagen promoter/CAT construct pMS3.5/CAT (5 μ g), trypsin-treated, pooled, replated, and treated with TGF- β 1 or TGF- β 2 (5 ng/ml) alone or in combination with OA (80 ng/ml) as indicated, for 30 h. Cells were then harvested and CAT activity was measured. The migration positions of acetylated (AC) and non-acetylated (C) [14 C]chloramphenicol are shown on the left. A representative CAT assay is shown.

was able to prevent transcriptional activation of pro α 2(I) collagen gene by TGF- β . Treatment of transiently transfected NIH-3T3 cells with TGF- β 1 or TGF- β 2 resulted in marked increase (3.7- and 6.1-fold respectively) in the activity of the pro α 2(I) collagen promoter/CAT construct (Figure 5). Interestingly, simultaneous treatment of cells with OA (80 ng/ml) potently prevented enhancement of pro α 2(I) collagen promoter by TGF- β 1 or TGF- β 2 (Figure 5).

DISCUSSION

Suppression of type I collagen gene expression is important in the prevention of tissue fibrosis. Previous studies have shown that glucocorticoids, as well as the cytokines IFN- γ and TNF- α , are potent inhibitors of type I collagen expression, and can abrogate the enhancement of type I collagen gene expression by TGF- β [5,7-9,42]. The results of the present study showed that OA, a specific inhibitor of cellular PP1 and PP2A, is a potent inhibitor of expression of type I and III procollagen genes by

human dermal fibroblasts. We also show that OA potently suppresses activity of the human pro α 2(I) collagen promoter/CAT construct in transiently transfected NIH-3T3 cells. Furthermore, OA prevents enhancement of type I and III collagen mRNA levels by TGF- β 2, and potently inhibits transcriptional activation of pro α 2(I) collagen gene by TGF- β . Our results are in accordance with, and extend, a recent observation that OA inhibits production of collagenase-sensitive proteins by fibroblasts [43].

These results provide evidence that PP1 and PP2A play an important role in maintaining type I and III collagen gene expression by dermal fibroblasts. An interesting observation was that expression of type I collagen mRNAs was reduced by OA in cells maintained both at 1 and at 10% FCS. However, the effect of OA on type I and III collagen mRNAs was clearly more potent in cells maintained in 1% FCS as compared with 10% FCS, indicating that growth factors in serum have an antagonistic effect against OA-elicited suppression of type I collagen gene expression. In accordance with previous findings on synovial cells and HT-1080 fibrosarcoma cells [28,29], OA induced expression of collagenase and stromelysin-1 mRNA abundance in dermal fibroblasts. These results show that selective inhibition of PP1 and PP2A results in an 'antifibrotic' phenotype of dermal fibroblasts characterized by simultaneous suppression of type I and III collagen, and induction of MMP gene expression.

Tissue-specific expression of pro α 1(I) and pro α 2(I) collagen genes is controlled by several regulatory *cis*-elements in the 5'-flanking regions of these genes [19-24]. To date, some of the *trans*-acting factors, including Sp-1 and NF-1, which interact with specific regulatory elements in these promoters, have been identified [4,22]. Previously, OA has been shown to induce expression of components of the AP-1 complex, including *c-jun*, *junB*, *junD* and *c-fos* in several types of cells [28,29,44]. In addition, induction of collagenase gene transcription by OA is dependent on expression of *c-jun*, *junB* and *c-fos*, depending on cell type [28,29]. However, the inhibitory effect of OA on type I and III collagen gene expression is not dependent on protein synthesis, as demonstrated by the cycloheximide experiment (Figure 2). It is therefore unlikely that the suppressing effect of OA on type I and III collagen is mediated by altered expression of *trans*-acting factors regulating expression of these genes. Thus it is likely that OA-elicited inhibition of type I and III collagen gene expression involves altered phosphorylation of pre-existing transcription factors, resulting in alterations in their DNA-binding activity or *trans*-activating capacity [25,26]. It has been shown that dephosphorylation of c-Jun by PP2A *in vivo* and *in vitro* enhances its binding activity to the AP-1 element [45,46], due to dephosphorylation of one threonine and two serine residues close to the DNA-binding domain [47].

In conclusion, the results presented here indicate that inhibition of activity of the cellular phosphoserine- and phosphothreonine-specific protein phosphatases, PP1 and PP2A, results in a marked suppression of type I collagen gene expression at the transcriptional level and inhibits transcriptional activation of type I collagen gene expression by TGF- β . It is conceivable that elucidation of the detailed molecular mechanisms of OA-elicited inhibition of type I collagen gene expression, especially the role of PP1 and PP2A in it, may offer possibilities for developing novel therapeutic modalities for disorders characterized by tissue fibrosis.

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