Journal of Cellular Biochemistry 76:354–359 (2000)

FAST TRACKS

Extracellular-Signal Regulated Kinase Signaling Pathway Mediates Downregulation of Type I Procollagen Gene Expression by FGF-2, PDGF-BB, and Okadaic Acid in Osteoblastic Cells

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Abstract Although basic fibroblast growth factor (FGF-2) had been shown to inhibit type I collagen gene expression in osteoblast, its inhibitory mechanism is unknown. In the present study, we investigated the underlying mechanisms by which growth factors downregulate type I collagen gene expression. Treatment of mouse osteoblastic MC3T3-E1 cells with okadaic acid (40 ng/ml), an inhibitor of phosphoserine/threonine-specific protein phosphatase and activator of ERK1/2, for 24 h and 48 h completely inhibited steady-state mRNA levels of type I collagen. FGF-2 (30 ng/ml), platelet-derived growth factor-BB (PDGF-BB), 30 ng/ml, and serum, which activate ERK mitogen-activated protein kinase (MAPK) pathway also inhibited collagen type I gene expression, suggesting that the activation of ERK pathway mediates inhibition of type I collagen mRNA. This observation was further confirmed by experiments using inhibitors of the ERK pathway (i.e., PD and U0126), which increased type I collagen mRNA in MC3T3-E1 cells, indicating that the inhibition of ERK pathway upregulates type I collagen gene expression. Low serum (0.3%) markedly increased type I collagen mRNA. MEK inhibitor PD inhibited c-fos induction by FGF-2 and PDGF-BB, suggesting that c-fos is the downstream target of ERK pathway. Our data have clearly demonstrated for the first time that the ERK MAPK pathway play an important role in the regulation of type I collagen gene expression in osteoblastic cells. Results also showed that one of the mechanisms by which FGF-2 and PDGF-BB downregulate type I collagen gene expression in the osteoblast is through the activation of ERK signaling pathway. J. Cell. Biochem. 76:354–359, 2000.

Key words: type I collagen; osteoblast; okadaic acid; FGF-2; PDGF; MAP kinase signaling pathway

Type I collagen, a heterotrimeric protein made up of two polypeptide chains, α1(I) and α2 (I), in the ratio 2:1, is the most abundant protein in the bone, accounting for approximately 90% of the organic matrix [Vuorio and de Crombrugghe, 1990; Termine and Robey, 1996]. Collagen type I is a product of the α1(I) and α2(I) genes localized on different chromosomes and is the major protein synthesized and secreted by the osteoblast (bone-forming cell). Increased type I collagen synthesis is directly related to the structural properties of bone whereas a decreased collagen content of bone is associated with increased bone fragility. Furthermore, an increased collagen synthesis and hypomineralization seen in osteoarthritic bone suggest that bone collagen metabolism is an important factor in the pathogenesis of osteoarthritis [Mansell and Bailey, 1998].

Although basic fibroblast growth factor (FGF-2) and platelet-derived growth factor (PDGF) play an important role in bone metabolism and that their regulation of osteoblastic cell proliferation and differentiation impacts in an important fashion on bone metabolism, the molecular mechanisms of action of these growth factors that enable them to functionally affect the osteoblast are not well understood. PDGF stimulates cell growth of normal human osteoblastic (HOB) cells [Zhang et al., 1991] and MC3T3-E1 cells [Okazaki et al., 1992]. FGF-2 is a potent mitogen for mesenchymal cells, in-

Grant sponsor: National Institutes of Health; Grant number: PO1 AR32087.

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Received 28 October 1999; Accepted 2 November 1999

This article published online in Wiley InterScience, January 2000.

cluding normal human bone marrow stromal cells [Robinson et al., 1995], osteoblasts [Rodan et al., 1989], as well as mouse MC3T3-E1 cells [Suzuki et al., 1996]. FGF-2 stimulates in vivo endosteal bone formation in rats [Mayahara et al., 1993; Nakamura et al., 1995] but inhibits collagen type I gene expression in MC3T3-E1 cells [Hurley et al., 1993; Boudreaux et al., 1996] demonstrating differences in in vivo and in vitro systems. Also, overexpression of FGF-2 as well as disruption of FGF receptor cause skeletal defects [Coffin et al., 1995; Deng et al., 1996]. Furthermore, effects of PDGF on type I collagen synthesis have been small, inconsistent and nonspecific to osteoblasts [Centrella et al., 1989; Canalis and Hock, 1994].

Cell surface receptors for FGF-2 and PDGF possess intrinsic protein tyrosine kinase activity and transduce signals from the cell surface to the nucleus by activating kinase cascades. In mammalian systems, the best characterized MAP kinase cascades are: (1) the extracellular signal-regulated kinase (ERK), which uses the Ras → Raf → MEK → ERK cascade, (2) c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and (3) p38 MAP kinase [Su and Karin, 1996; Robinson and Cobb, 1997]. The common pathway is a three-kinase cascade that is sequentially activated involving the activation of MAP kinase kinase kinase (MAPKKK/MEKK)/Raf-1, then activated Raf-1 phosphorylates and activates MAP kinase kinase (MAPKK/MEK), which in turn phosphorylates and activates downstream ERK1 and ERK2 [Su and Karin, 1996; Robinson and Cobb, 1997]. In a similar fashion, cytokines and stress factors primarily activate JNK/SAPK and p38 MAP kinase cascades [Waskiewicz and Cooper, 1995; Su and Karin, 1996]. We have recently demonstrated that FGF-2 and PDGF-BB activate the ERK MAPK cascade in osteoblastic cells [Chaudhary and Avioli, 1997, 1998a].

One of the goals of our research is to understand how growth factors modulate bone matrix proteins gene expression, with particular emphasis on defining the mechanisms(s) by which FGF-2 and PDGF affect collagen type I gene expression. Thus, in the present study, we evaluated the role of the ERK MAPK pathway in collagen type I gene expression since both these growth factors activate ERK signaling pathway in osteoblastic cells. Results have demonstrated that the activation of ERK pathway by FGF-2, PDGF-BB, serum, and okadaic acid downregulates type I collagen gene expression in osteoblastic cells.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS), α-minimum essential medium (α-MEM), Dulbecco's phosphate-buffered saline (PBS), ascorbic acid, and β-glycerophosphate were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human FGF-2 and PDGF-BB were obtained from R&D Systems (Minneapolis, MN). Okadaic acid and PD98059 were purchased from Calbiochem (San Diego, CA) and MEK inhibitor U0126 from Promega (Madison, WI). All other chemicals were of analytical or molecular biology grade.

Cell Culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [Sudo et al., 1983] were cultured in α-minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS) and maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells (2×10^6) were seeded in P-100 tissue culture dishes, grown for 2–3 days until confluent, and then treated with the desired agents for specified time periods.

Northern Blot Analysis

For the preparation of total cellular RNA, cells were washed with phosphate-buffered saline (PBS) three times and immediately lysed in harvest buffer composed of 1% sodium dodecyl sulfate (SDS), 0.25% NaCl, 30 mM Tris (pH 8.0), 2 mM EDTA (pH 8.0), and 1 mg/ml proteinase K. After 30 min of incubation at 37°C, the cell lysates were extracted with phenol/chloroform and then with chloroform as described previously [Chaudhary and Avioli, 1996]. The total RNA was further purified by precipitation in 2 M LiCl and ethanol, dissolved in diethylpyrocarbonate (DEPC)-treated water, and quantified by measuring absorbance at 260/280 nm on a Beckman spectrophotometer. Aliquots of total RNA (20 μg) were separated under denaturing conditions on 1% agarose/formaldehyde gel, using standard protocols as described [Chaudhary and Avioli, 1996] and stained with ethidium bromide to confirm RNA integrity and assess amounts of loading. RNA was trans-

ferred by downward alkaline capillary diffusion [Chomczynski, 1992] onto nylon membranes (Schleicher & Schuell, Keene, NH). The blots were dried for 1 h at 80°C under vacuum and prehybridized in Hybrisol I (Oncor, Gaithesburg, MD) for 6 h at 42°C and then hybridized overnight at 42°C with [^{32}P]labeled human procollagen α1 (I) cDNA probe generated by random priming labeling. After hybridization, blots were washed twice with 2× SSC/0.1% SDS for 15 min at room temperature and then twice with 0.2× SSC/0.1% SDS for 15 minute at 50°C. Blots were exposed to Amersham Hyperfilm-MP at −80°C for appropriate time, and the film was developed using a Kodak RP X-OMAT processor. The data presented are representative of two or three individual experiments with similar results.

Densitometric Analysis

Northern blot autoradiographs were scanned and quantitated by using a computerized ISS SepraScan 2001 (Integrated Separation Systems-Enprotech, Natick, MA). Membranes were hybridized to human procollagen α1 (I) probe, then stripped and reprobed with the rat GAPDH [^{32}P]labeled cDNA probe. The density readings of procollagen α1 (I) mRNA were normalized to the respective GAPDH mRNA density readings.

RESULTS

Effects of PD98059 (PD), Okadaic Acid (OA), and Ascorbic Acid (AA) Plus β-Glycerophosphate (βGP) on α1(I) Procollagen mRNA in MC3T3-E1 Cells

To determine whether activation of ERK pathway regulates type I collagen gene repression, MC3T3-E1 cells were cultured in the α-MEM medium containing 10% FBS. Cells were treated with PD (100 μM), a specific inhibitor of MEK [Alesi et al., 1995], or okadaic acid (40 ng/ml), a potent inhibitor of serine/threonine protein phosphatases 1 and 2A (PP1 and PP2A) and activator of ERK1/2 [Amaral et al., 1993; Rondinone et al., 1996], for 24 h and 48 h. Total cellular RNA were analyzed by Northern blot analysis. Type I collagen mRNA levels were increased approximately 4-fold in PD-treated cells, as compared with control cells at both 24 h and 48 h (Fig. 1), as determined by densitometric analysis. By contrast, okadaic acid (OA) completely inhibited collagen type I mRNA levels at both 24 h and 48 h. Since ascorbic acid (AA) and

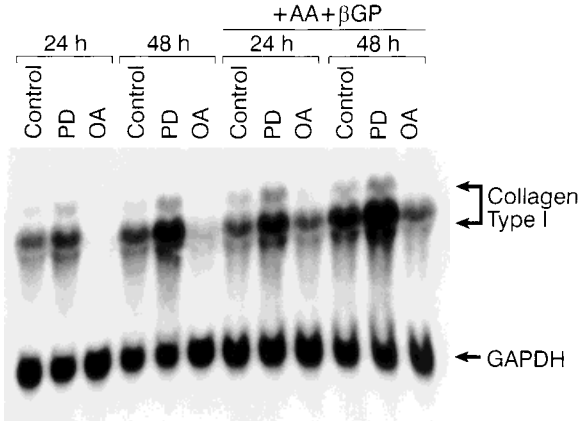

Fig. 1. Effects of MEK inhibitor PD98059 (PD), okadaic acid (OA), and ascorbic acid (AA) plus β-glycerophosphate (βGP) on α1(I) procollagen mRNA in mouse osteoblast-like MC3T3-E1 cells. Cells were treated with PD (100 μM) or OA (40 ng/ml) in the presence or absence of AA (50 μg/ml) plus βGP (10 mM) for 24 h and 48 h. Total cellular RNA (20 μg) was analyzed by Northern blot analysis.

β-glycerophosphate (βGP) modulate type I collagen synthesis, we also determined the effects of PD and OA in the presence of AA plus βGP. As shown in Figure 1, type I collagen mRNA levels were decreased by OA and increased by PD both in the presence and absence of AA plus βGP. The addition of AA and βGP increased type I collagen mRNA by approximately 75% (determined by densitometric analysis), as compared with nontreated control cells.

Effects of Serum, FGF-2, and PDGF on α1(I) Procollagen mRNA

Since serum activates ERK MAP kinase pathway that inhibits type I collagen synthesis, we determined whether very low serum (0.3%) in the medium would increase type I collagen gene expression. MC3T3-E1 cells were cultured in 0.3% and 10% serum and also treated with FGF-2 and PDGF-BB for 24 h. As shown in Figure 2, type I collagen mRNA levels were increased (approximately 10-fold determined by densitometric analysis) in cells grown in low serum (0.3%), as compared with cells grown in 10% serum. Both FGF-2 and PDGF-BB inhibited type I collagen gene expression at 10% and 0.3% serum concentrations.

Comparison of Effects of Two Different MEK Inhibitors U0126 and PD on α1(I) Procollagen mRNA

Since PD inhibits the activation of nonphosphorylated MEK and is less soluble in aqueous

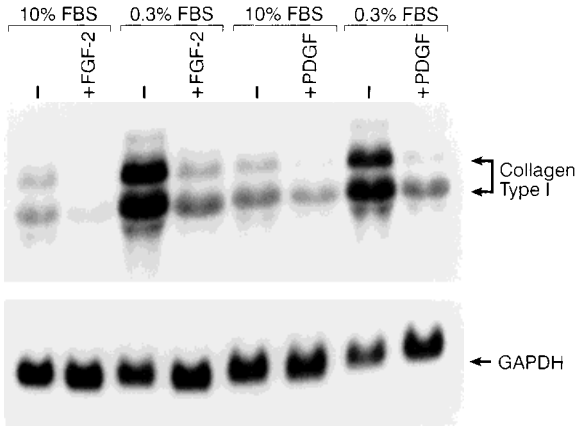

Fig. 2. Effects of serum, basic fibroblast growth factor (FGF-2), and platelet-derived growth factor (PDGF) on α1(I) procollagen mRNA. MC3T3-E1 cells were cultured in α-MEM medium containing AA (50 μg/ml) plus βGP (10 mM) and 10% or 0.3% FBS in the presence or absence of FGF-2 (30 ng/ml) or PDGF-BB (30 ng/ml) for 24 h. Total cellular RNA (20 μg) was analyzed by Northern blot analysis.

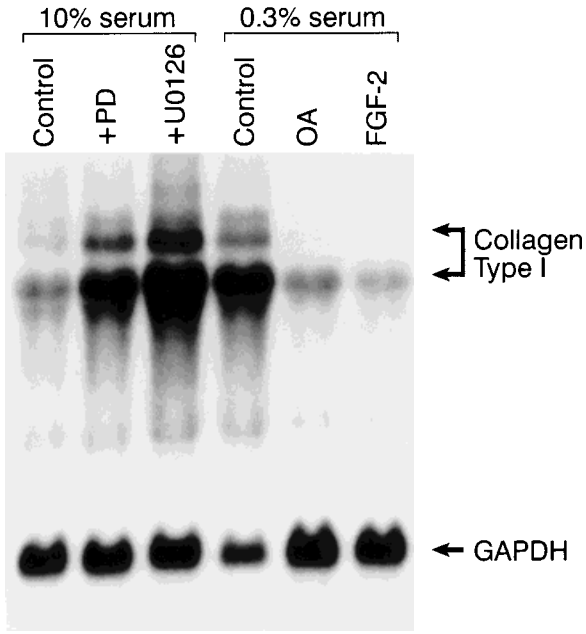

Fig. 3. Comparison of effects of MEK inhibitors U0126 and PD on α1(I) procollagen mRNA. MC3T3-E1 cells were cultured in α-MEM medium containing AA (50 μg/ml) plus βGP (10 mM) and 10% or 0.3% fetal bovine serum (FBS). Cells were treated with PD (100 μM), U0126 (20 μM), or basic fibroblast growth factor (FGF-2) (30 ng/ml) for 24 h. Total cellular RNA (20 μg) was analyzed by Northern blot analysis.

medium [Alesi et al., 1995], we investigated the effect of a recently reported more potent and selective inhibitor of MEK, U0126 [Favata et al., 1998]. As shown in Figure 3, both PD and U0126 increased type I collagen mRNA but U0126 was more potent than PD. On the other hand, OA and FGF-2 inhibited type I collagen mRNA stimulated by low serum (0.3%).

Effect of PD on c-fos mRNA Induction by FGF-2 and PDGF

Since serum, FGF-2, and PDGF induce expression of c-fos and activate the ERK MAPK pathway, we examined whether PD would inhibit c-fos induction by FGF-2 and PDGF-BB. As shown in Figure 4, treatment of MC3T3-E1 cells with PD inhibited c-fos induction by FGF-2 and PDGF-BB. The induction of c-fos by FGF-2 and PDGF-BB was decreased more than 70% in the presence of PD, as determined by densitometric analysis.

DISCUSSION

In the present study, we investigated the mechanism(s) by which FGF-2 and PDGF inhibit type I collagen gene expression. Our results have demonstrated for the first time that the ERK MAPK pathway mediates the inhibition of type I collagen gene expression by FGF-2, PDGF, and serum. FGF-2 and PDGF-BB independently inhibited type I collagen gene expression in both 10% or 0.3% serum. Our data are in agreement with previous observations that FGF-2 inhibits type I collagen gene expression in osteoblastic cells [Hurley et al., 1993; Boudreaux et al., 1996]. Our data also showed that okadaic acid, a specific inhibitor of serine/threonine protein phosphatases 1 and 2A (PP1 and PP2A) and activator of ERK1/2 [Amaral et al., 1993; Rondinone et al., 1996] potently suppressed type I collagen mRNA levels in osteoblastic cells. Similarly, okadaic acid had been shown to inhibit type I collagen gene expression in human skin fibroblasts and mouse NIH 3T3 cells [Westermarck et al., 1995; Wang and Raghow, 1996]. These results provide evidence that protein phosphorylation and dephosphorylation play an important role in regulating type I gene expression in osteoblast. An interesting observation was that the expression of type I collagen mRNA was markedly increased by low serum (0.3%). This observation is the first to demonstrate that very low levels of serum can increase type I collagen gene expression in osteoblastic cells.

Our results have clearly demonstrated that activation of the ERK MAPK pathway downregulates type I collagen gene expression. This

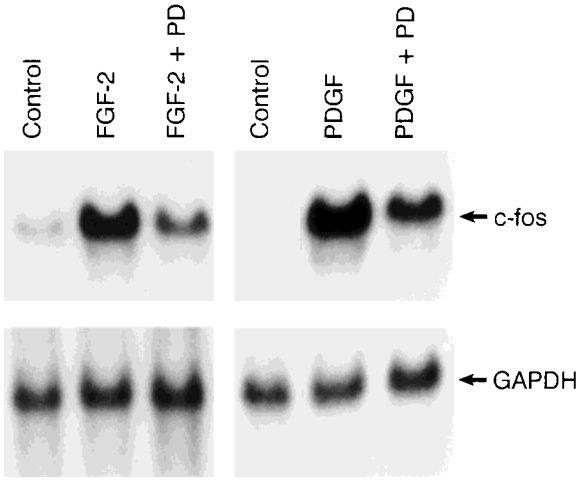

Fig. 4. Effect of PD on c-fos mRNA induction by basic fibroblast growth factor (FGF-2) and PDGF. MC3T3-E1 cells were made quiescent in α-MEM medium containing 0.3% serum for 48 h. Cells were pretreated with PD (100 μM) for 30 min, then FGF-2 (30 ng/ml) or platelet-derived growth factor-BB (PDGF-BB) (30 ng/ml) was added for additional 30 min. Total cellular RNA (20 μg) was analyzed by Northern blot analysis.

was further confirmed by our experimental data demonstrating that the inhibition of ERK1/2 by specific and selective inhibitors of MEK U0126 and PD upregulated type I collagen gene expression. We have recently reported that FGF-2 and PDGF do not activate JNK/SAPK pathway in osteoblastic cells [Chaudhary and Avioli, 1998b]. Taken together, our data support the concept that ERK pathway mediates inhibitory effects of FGF-2, PDGF-BB and serum on type I collagen gene expression. These results are consistent with observations that overexpression of oncogenic Ras markedly inhibited type I collagen gene expression in Rat 1 fibroblasts [Slack et al., 1992]. By contrast, inhibition of MAPK by dominant negative mutants of MAPK inhibited type I collagen gene expression in rat hepatic stellate cells [Davis et al., 1996], suggesting that ERK pathway had a positive effect on type I collagen gene expression. The reason for these discrepant results remains unclear. However, these differences could be attributable to different cell types, possibly bifurcation of signaling pathway(s) and differences in the experimental methods used as transiently transfected cells were used in these experiments [Slack et al., 1992, Davis et al., 1996].

Among the consequences of ERK pathway activation are changes in the transcription of key growth-regulated genes such as the immediate-early genes c-fos and c-jun. One of the best characterized ERK-responsive promoters is that of the c-fos gene [Price et al., 1996; Su and Karin, 1996; Hodge et al., 1998; Seternes et al., 1998]. The ERK-stimulated transcription of c-fos requires an enhancer sequence within its promoter known as the serum response element (SRE), and is mediated by serum response factor (SRF) and one of the ternary complex factors (TCFs). The phosphorylation of the TCF Elk-1 by ERK1/2 leads to their high-affinity interaction in the ternary complex and a concomitant increase in transcriptional activation. The activation of Elk-1 stimulates SRE of c-fos promoter resulting in the increased transcription of c-fos [Price et al., 1996; Su and Karin, 1996; Hodge et al., 1998; Seternes et al., 1998]. Our results have shown that FGF-2 and PDGF-BB induced c-fos gene expression, which was inhibited by PD98059, suggesting that c-fos is the downstream target of ERK pathway in MC3T3-E1 cells. Thus, one of the mechanisms by which ERK activation may inhibit type I collagen gene expression could be via induction of c-fos by FGF-2, PDGF-BB, and serum, as the overexpression of c-fos in MC3T3-E1 cells has been shown to inhibit type I collagen [Kuroki et al., 1992].

ACKNOWLEDGMENTS

This research was supported in part by NIH grant PO1 AR32087 (to L.V.A.).

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