Basic fibroblast growth factor destabilizes osteonectin mRNA in osteoblasts

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Delany, Anne M., and Ernesto Canalis. Basic fibroblast growth factor destabilizes osteonectin mRNA in osteoblasts. Am. J. Physiol. 274 (Cell Physiol. 43): C734–C740, 1998.—Osteonectin (secreted protein acidic and rich in cysteine, 40-kDa basement membrane) is a glycoprotein abundantly expressed in bone and in other tissues undergoing active remodeling. Fibroblast growth factors (FGFs) are important in skeletal development and fracture repair, events associated with extracellular matrix remodeling. We used the murine osteoblastic cell line MC3T3 to determine whether basic FGF (bFGF) regulates osteonectin expression in bone. Northern blot analysis showed that bFGF decreased osteonectin transcripts in a dose- and time-dependent manner. This regulation was independent of the mitogenic effect of bFGF but was dependent on new protein synthesis. Immunoprecipitation of [35S]methionine-cysteine osteoblast-conditioned medium and cell layer proteins showed that bFGF decreased osteonectin synthesis. Nuclear runoff assays failed to reveal regulation of osteonectin gene transcription by bFGF. However, bFGF dramatically decreased the stability of osteonectin mRNA in transcriptionally arrested osteoblasts. This destabilization of osteonectin mRNA may be one means by which bFGF regulates extracellular matrix remodeling.

OSTEONECTIN, or secreted protein acidic and rich in cysteine (SPARC), is a modular glycoprotein that is expressed in bone and in other tissues undergoing active remodeling. Although the precise role of osteonectin in bone and other tissues has not been defined, in vitro studies suggest that osteonectin has pleiotropic effects on gene expression (22). Osteonectin induces metalloproteinase expression in fibroblasts and stimulates angiogenesis in vitro and in vivo (17, 20, 41). In addition, osteonectin may localize the activities of growth factors and proteases within the matrix, because osteonectin binds type I collagen and platelet-derived growth factor (PDGF) B chains, thrombospondin, and plasminogen (19, 22, 32). Osteonectin limits the function of PDGF-BB and PDGF-AB by inhibiting their binding to fibroblasts (32). Similarly, osteonectin inhibits the basic fibroblast growth factor (bFGF)-induced migration and proliferation of endothelial cells, although not through a direct interaction with the growth factor (15).

Much of the work on the function of osteonectin has been done using nonskeletal cells, and some of the effects of osteonectin are cell type specific. For example, osteonectin can disrupt cell spreading only in selected cell cultures (21). Similarly, in endothelial cells, osteonectin decreases DNA synthesis by delaying the onset of S phase, whereas data from osteoblastic cells suggest that osteonectin has a modest stimulatory effect on cell proliferation (10, 45).

In bone, osteonectin is among the most abundant noncollagenous extracellular matrix proteins, and the skeleton is one of the richest sources of the protein. Osteonectin is synthesized by cells of the osteoblastic lineage; binds hydroxyapatite, calcium, and type I collagen; and inhibits mineralization in vitro (7, 19, 34). Osteonectin expression is decreased in osteoblasts derived from patients with osteogenesis imperfecta, and osteonectin mRNA is decreased in osteoblasts derived from the flox/flox mouse, an animal model for some forms of bone fragility (8, 28). These data suggest that osteonectin modulates matrix organization and mineralization. This concept is further supported by the finding that, in rodent osteoblastic cells induced to form nodules in vitro, osteonectin expression increases as the matrix matures and mineralizes (3). In vivo studies suggest that osteonectin plays a role in development, because injection of anti-SPARC antibodies into Xenopus embryos results in developmental defects (30). Similarly, overexpression of osteonectin in transgenic nematodes causes developmental defects, suggesting that the appropriate and regulated expression of this matrix protein may be essential for normal development (36).

bFGF is a potent mitogen for cells of the osteoblastic lineage, and it represses the differentiated phenotype of mature osteoblasts (6). FGFs are important in skeletal development and fracture repair, events associated with active extracellular matrix remodeling (18, 43). Because osteonectin is expressed in areas of matrix remodeling, its coordinated and temporally appropriate expression may be important for the development of a transitional matrix and regulated angiogenesis, which are essential in development and fracture repair. This regulation may be mediated through the actions of growth factors and cytokines, and we postulated that bFGF regulates osteonectin expression in osteoblasts.

MC3T3-E1 is a clonal osteogenic cell line derived from neonatal mouse calvaria. These cells are well characterized and provide a homogeneous source of osteoblastic cells for study. They express high levels of alkaline phosphatase and differentiate into osteoblasts that can form calcified bone tissue in vitro (3, 39). The response of MC3T3-E1 cells to many growth factors and hormones mimics that of primary cultures of rodent osteoblastic cells. Therefore, using cultures of MC3T3-E1 cells, we examined the regulation of osteonectin by bFGF and initially characterized its mechanisms of action.
MATERIALS AND METHODS

Cell cultures. Early-passage MC3T3-E1 osteoblasts were cultured in α-minimum essential medium (GIBCO BRL, Grand Island, NY) containing 20 mM N-2-hydroxyethylpipera-
zine-N'-2-ethane sulfonic acid (HEPES) and 10% fetal bovine serum (Summit Biotechnologies, Ft. Collins, CO) (39). Cells were plated at a density of ~14,000 cells/cm² and were grown to confluence (~130,000 cells/cm²) after 4 days of culture. Cultures were then rinsed and transferred to serum-free medium containing 0.1% bovine serum albumin (Fluka Chemical, Ronkonkoma, NY) and 50 µg/ml ascorbic acid for 24 h. At the time of serum deprivation, the cells were considered to be in the matrix-deposition phase of the osteoblast culture. The cells were then exposed to test or control medium in the absence of serum for 2–48 h. After 48 h, cultures treated with βFGF had approximately twofold more cells than the untreated controls.

Primary cultures of mouse osteoblastic cells were isolated from the parietal bones of neonatal mice (23). This procedure was approved by the Institutional Animal Care and Use Committee of Saint Francis Hospital and Medical Center. Parietal bones, dissected free of sutures, were subjected to five sequential 15-min digestions with bacterial collagenase (CLS II; Worthington Biochemical, Freehold, NJ). Cells harvested from digestes 3-5 were cultured as a pool at an initial plating density of ~10,000 cells/cm². These cells have been demonstrated to have osteoblastic characteristics (Ref. 23 and Delany, unpublished data). Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with nonessential amino acids, 20 mM HEPES, 100 µg/ml ascorbic acid, and 10% fetal bovine serum. When the cells reached confluence (~1 wk after plating), they were rinsed and transferred to serum-free medium for 24 h and then exposed to test or control medium for 2–48 h.

Cycloheximide and 5,6-dichlorobenzimidazole riboside (DRB) (Sigma, St. Louis, MO) were dissolved in absolute ethanol, and, at dilutions of <1:10,000, an equal amount of ethanol was added to control cultures. Hydroxyurea (Sigma) and βFGF (Austral, San Ramon, CA) were dissolved in culture medium.

Northern blot analysis. Total cellular RNA was isolated with guanidine thiocyanate, at acid pH, followed by a phenol-
chloroform (PC) extraction as described (4). Equal amounts of RNA (10 µg) were denatured and subjected to electrophore-
sis through formaldehyde-agarose gels, and the RNA was blotted onto GeneScreen Plus as directed by the manufac-
turer (NEN Life Sciences Products, Wilmington, DE). Restriction fragments containing a 1.5-kilobase (kb) bovine osteonec-
tin cDNA (1) (provided by M. Young, Bethesda, MD) and a 750-base pair (bp) murine 18S RNA cDNA (28) (American Type Culture Collection, Rockville, MD) were labeled with [α-32P]dCTP (specific activity 3,000 Ci/mmole; NEN Life Sci-
ces Products) by random-primed second-strand synthesis (Prime-A-Gene kit, Promega, Madison, WI). Hybridizations were carried out at 42°C in 50% formamide, 750 mM sodium chloride-50 mM sodium phosphate-5 mM EDTA, 5× Den-
hartig’s solution, and 0.4% sodium dodecyl sulfate (SDS). Posthybridization washes were performed a total of 35°C in 150 mM sodium chloride-15 mM sodium citrate and 0.1% SDS. Appropriate exposures of the autoradiograms were analyzed by densitometry, and osteonectin RNA levels were normalized to those of 18S rRNA. The nuclear runoff assay shown is representative of two experiments.

Statistical analysis. Differences in the slopes of RNA decay curves were analyzed by the method of Sokal and Rohlf (38).

RESULTS

MC3T3 cells produce a single osteonectin transcript of ~2.2 kb. Treatment of serum-deprived MC3T3 cells...
with 30 ng/ml bFGF caused a time-dependent decrease in osteonectin mRNA (Fig. 1). Northern blot analysis and densitometry showed that after 12 h of treatment with bFGF, osteonectin transcripts were decreased by ~50%, and after 24 h, bFGF decreased osteonectin transcripts by ~75%. The same level of inhibition was observed after 48 h of treatment. Similarly, bFGF decreases osteonectin mRNA in primary cultures of mouse osteoblastic cells, confirming that the effect is not a cell line-specific phenomenon (Fig. 2).

Downregulation of osteonectin mRNA by bFGF was dose dependent, because bFGF at 1–50 ng/ml decreased osteonectin transcripts by 30–90% after 24 h (Fig. 3). Immunoprecipitation of [35S]methionine-[32P]cysteine-labeled osteoblast-conditioned medium and cell layer proteins showed that bFGF decreased the synthesis of osteonectin by 40–60% after 24 and 48 h of treatment (Fig. 4). In the conditioned medium, the primary osteonectin species detected by the anti-SPARC antiserum had an apparent molecular mass of 38 kDa, and this protein had the same mobility as osteonectin purified from bovine bone. The cell layer contained multiple osteonectin species with apparent molecular masses of ~41, 38, and 29 kDa, and a less-abundant 34-kDa form. Nonimmune serum did not immunoprecipitate radiolabeled proteins (data not shown).

To determine whether the effect of bFGF on osteonectin mRNA expression was dependent on new protein synthesis, osteoblasts were treated with bFGF in the presence or absence of 2 µg/ml cycloheximide for 24 h. This dose of cycloheximide inhibited protein synthesis by 85–90% after 24 h of treatment, yet the cells were >95% viable as evaluated by trypsin blue staining. Cycloheximide alone decreased osteonectin mRNA levels and prevented the repression of osteonectin transcripts by bFGF (Fig. 5). To determine whether downregulation of osteonectin expression by bFGF was dependent on cell replication, the DNA synthesis inhibitor hydroxyurea was used. In serum-starved MC3T3 cells, hydroxyurea at 1 mM abolished the proliferative effect of bFGF (data not shown), but hydroxyurea had no effect on the repression of osteonectin transcripts by bFGF (Fig. 6). These data indicate that the repression of osteonectin expression by bFGF is protein synthesis dependent and independent of the ability of bFGF to stimulate cell replication.

To determine whether bFGF modified the stability of osteonectin mRNA in osteoblasts, the RNA polymerase II-specific inhibitor DRB was used to arrest transcription, and the decay of osteonectin mRNA was monitored by Northern blot analysis (46). Serum-deprived cultures were treated with control medium or with 30 ng/ml bFGF for 6 h and then exposed to 72 µM DRB for up to 24 h. Although treatment of MC3T3 cells with DRB for 24 h decreased protein synthesis ~70%, the cells remained >95% viable as evaluated by trypan blue staining. In transcriptionally arrested osteoblasts, the half-life of osteonectin mRNA was ~24 h, but in the presence of bFGF, the half-life of the transcript decreased to ~10 h (Fig. 7). In contrast, bFGF increased the stability of glyceraldehyde-3-phosphate dehydrogenase mRNA in transcriptionally arrested cells (data not shown). To determine if there was a transcriptional component to the regulation of osteonectin by bFGF, nuclear runoff assays were performed. bFGF did not alter the rate of osteonectin gene transcription at 2 (not shown), 6, 24, or 48 h of treatment (Fig. 8). However, bFGF did regulate collagenase 3 transcription, confirming growth factor activity (Fig. 8, Ref. 16, and Delany, 1995).

Fig. 2. Effect of bFGF at 100 ng/ml on osteonectin mRNA expression in primary mouse osteoblastic cells treated for 8 or 24 h. Total RNA from control (−) or bFGF-treated (+) cultures was subjected to Northern blot analysis and hybridized with a 32P-labeled bovine osteonectin cDNA (ON); blot was stripped and hybridized with a labeled rat 18S RNA cDNA. Transcripts were visualized by autoradiography; osteonectin mRNA is shown at top, and 18S RNA is shown at bottom. Similar results were obtained when cells were treated with 30 ng/ml bFGF. These results are representative of 3 cultures.

Fig. 3. Effect of bFGF at 1–50 ng/ml on osteonectin mRNA expression in MC3T3 cells treated for 24 h. Total RNA from MC3T3 cells was subjected to Northern blot analysis and hybridized with 32P-labeled bovine osteonectin cDNA (ON); blot was stripped and hybridized with a labeled rat 18S RNA cDNA. Transcripts were visualized by autoradiography; osteonectin mRNA is shown at top, and 18S RNA is shown at bottom. These results are representative of 3 cultures.
unpublished data). After 6 h of treatment, bFGF caused a modest decrease in collagenase 3 transcription, followed by an approximately twofold increase in collagenase 3 transcription seen after 24 or 48 h. These data indicate that bFGF decreases osteonectin expression by decreasing transcript stability, and that changes in gene transcription do not play a role in the regulation of osteonectin by bFGF.

**DISCUSSION**

FGFs are potent regulators of gene expression in cells of the osteoblastic lineage, playing a role in skeletal development and fracture repair (18, 43). Osteoblasts synthesize FGFs, which can be stored in the extracellular matrix (11). bFGF is mitogenic for cells of the osteoblastic lineage and represses the differentiated function of mature osteoblasts (6). Our data demonstrate that bFGF downregulates osteonectin synthesis in cultured osteoblasts, further supporting this premise. bFGF was effective at a dose as low as 1 ng/ml, with a maximal effect at 50 ng/ml. These doses of bFGF also stimulate DNA synthesis and inhibit alkaline phosphatase activity and type I collagen expression in osteoblastic cells (6, 24). It is important to note that osteonectin itself can regulate the cell cycle (10, 45). However, experiments utilizing the DNA synthesis inhibitor hydroxyurea show that the downregulation of osteonectin expression by bFGF is independent of the mitogenic properties of the growth factor. Therefore, the inhibition of osteonectin is not simply due to stimulation of cell replication.

Osteoblast-conditioned medium and cell layer, in particular, contain multiple osteonectin species. The lower-molecular-weight species are most likely proteolytic fragments of osteonectin. Osteonectin can be cleaved by plasmin and a number of metalloproteinases, including collagenase 3, stromelysin 1, and gelatinases A and B; and osteoblasts synthesize and secrete collagenase 3, gelatinases A and B, and stromelysin 3.

**Fig. 4.** Effect of bFGF at 30 ng/ml on osteonectin synthesis in MC3T3 cells treated for 24 or 48 h. Samples of conditioned medium corresponding to equal amounts of cell protein and equal amounts of cell layer proteins from [35S]methionine-[35S]cysteine-labeled control (−) or bFGF-treated (+) cultures were immunoprecipitated with anti-osteonectin antiserum. Immunoprecipitates were fractionated by polyacrylamide gel electrophoresis and visualized by fluorography. Molecular mass standards, shown on right, are in kDa. Arrow marks mobility of osteonectin purified from bovine bone. These results are representative of 3 cultures.

**Fig. 6.** Effect of bFGF at 30 ng/ml in presence or absence of hydroxyurea at 1 mM on osteonectin mRNA expression in MC3T3 cells treated for 24 h. Total RNA from untreated cells or cells cultured with bFGF or hydroxyurea was subjected to Northern blot analysis and hybridized with a [32P]-labeled bovine osteonectin cDNA (ON); blot was stripped and hybridized with a labeled rat 18S rRNA cDNA. Transcripts were visualized by autoradiography; osteonectin mRNA is shown at top, and 18S RNA is shown at bottom. These results are representative of 3 cultures.

**Fig. 5.** Effect of bFGF at 30 ng/ml in presence or absence of cycloheximide (cyclohex) at 2 µg/ml on osteonectin mRNA expression in MC3T3 cells treated for 24 h. Total RNA from untreated cells or cells cultured with bFGF or cycloheximide was subjected to Northern blot analysis and hybridized with a [32P]-labeled bovine osteonectin cDNA (ON); blot was stripped and hybridized with a labeled rat 18S rRNA cDNA. Transcripts were visualized by autoradiography; osteonectin mRNA is shown at top, and 18S RNA is shown at bottom. These results are representative of 3 cultures.

**Fig. 7.** Effect of bFGF at 30 ng/ml on osteonectin mRNA half-life in transcriptionally arrested MC3T3 cells. Confluent cultures were serum deprived and exposed to bFGF or control medium for 6 h before addition of 72 µM 5,6-dichlorobenzimidazole riboside (DRB). At selected times after addition of DRB, total RNA from control or bFGF-treated cultures was subjected to Northern blot analysis with [32P]-labeled bovine osteonectin cDNA. Osteonectin mRNA was visualized by autoradiography and quantitated by densitometry. Values are means ± SE for 3 cultures. Slope for DRB = −0.93, slope for DRB + bFGF = −3.71 (P < 0.01).
Late ribonucleases or RNA stabilizing factors. These findings were confirmed by transient transfection of MC3T3 cells with bovine osteonectin promoter fragments linked to the reporter gene chloramphenicol acetyl transferase or beta-galactosidase (Delany, unpublished data). In contrast, the half-life of osteonectin transcripts was shortened from >24 h in control osteoblasts to ~10 h in osteoblasts treated with bFGF. The data on the half-life of osteonectin mRNA in control cells agree with those obtained by other investigators utilizing fibroblastic cells (42). However, the mechanisms by which bFGF destabilizes osteonectin transcripts remain uncharacterized. Although there has been great progress in understanding how gene transcription is regulated, the mechanisms regulating eukaryotic mRNA stability are still largely unexplored. It is known that the 5’-cap structure and the poly-A tail play a role in protecting mRNA from exonucleolytic degradation; however, the sequences or structures that protect or target an RNA for endonucleolytic cleavage are less defined (12). RNA binding proteins may recognize specific sequences or specific secondary structures, such as stem loops. Frequently, sequences that regulate transcript stability are found within the 3′-untranslated region (UTR) (12). The osteonectin 3′-UTR is ~1 kb long and is composed of most of exon 10 of the gene (25). The coding region of osteonectin is well conserved across species, and the 3′-UTR of osteonectin also appears to be well conserved. The 3′-UTR of the bovine, mouse, and human transcript has regions that share >80% homology (1, 25, 44). Two of these regions are >100 bases long, suggesting conservation of functionally relevant sequences. Modeling of the 3′-UTR of the mouse osteonectin transcript, using the Zucker-Steigler algorithm, shows potential for extensive secondary structure (47). The long half-life of the osteonectin mRNA may be mediated in part by secondary structures within the 3′-UTR, which could protect the transcript from nucleolytic cleavage.

In general, there is little information about the mechanisms by which growth factors regulate osteonectin mRNA and peptide levels (2, 22, 29, 37, 42, 45). It is possible that other growth factors in addition to bFGF regulate osteonectin expression by posttranscriptional mechanisms. For example, PDGF-BB decreases osteonectin mRNA in MC3T3 cells with a time course similar to that observed for bFGF; however, PDGF-BB is not as potent as bFGF in this regard. It is interesting to note that osteonectin can antagonize selected effects of PDGF-BB and bFGF in fibroblasts and endothelial cells, respectively (15, 32). The ability of osteonectin to modulate the activities of bFGF and PDGF-BB, coupled with the ability of these growth factors to downregulate osteonectin expression suggests a possible feedback mechanism of regulation in tissues and in remodeling events in which osteonectin and these growth factors are coexpressed.

Data on the effects of osteonectin on angiogenesis, metalloproteinase expression, cell proliferation, and cell matrix interactions support the concept that osteo-

### Fig. 8. Effect of bFGF on osteonectin gene transcription in MC3T3 cells

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This suggests that a labile protein is involved in the maintenance of osteonectin mRNA levels. Such a factor may play a role in stabilizing osteonectin mRNA or in the transcription of the osteonectin gene. In cells cotreated with bFGF and cycloheximide, osteonectin RNA levels were modestly higher than those found in cells treated with cycloheximide alone. From these experiments, it is difficult to evaluate the significance of this effect. However, these data indicate that the downregulation of osteonectin by bFGF requires new protein synthesis, because the growth factor could not further repress osteonectin mRNA in the presence of cycloheximide.

It is possible that the repression of osteonectin mRNA by bFGF is mediated by c-jun unregulated proteins. bFGF induces c-jun expression, and in rat embryonic fibroblasts that overexpress c-jun, osteonectin mRNA is downregulated (26). Although the mechanisms by which c-jun decreases osteonectin expression have not been determined, studies suggest that its action is indirect. Evidence for this includes a late time course of action and the finding that the c-jun-overexpressing fibroblasts secrete a factor or factors that downregulate osteonectin transcripts (26). Because bFGF destabilizes osteonectin mRNA by an indirect mechanism, the effect may be mediated by a J un-regulated protein intermediate which could regulate ribonucleases or RNA stabilizing factors.
nectin is important in development, wound healing, and matrix remodeling (25). Growth factors and cytokines are important mediators in these processes. The regulation of osteonectin by growth factors and cytokines would modulate the extracellular matrix composition and, in turn, modulate gene expression. In conclusion, bFGF decreases osteoblast osteonectin expression by decreasing the stability of its transcript. Further characterization of the mechanisms by which bFGF destabilizes osteonectin transcripts may help to define the role of bFGF and osteonectin in matrix remodeling.

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