Basic Fibroblast Growth Factor Inhibits Type I Collagen Gene Expression in Osteoblastic MC3T3-E1 Cells*

(Received for publication, June 3, 1992)

Marja M. Hurley‡, Christine Abreu, John R. Harrison, Alexander C. Lichtler§, Lawrence G. Ruisz, and Barbara E. Kream

From the Departments of Medicine and §Pediatrics, The University of Connecticut Health Center, Farmington, Connecticut 06030-1850

We examined the effect of basic fibroblast growth factor (bFGF) on a1(I) procollagen mRNA levels, a1(I) collagen gene transcription, and a1(I) collagen promoter activity in osteoblastic MC3T3-E1 cells. Cells were stably transfected with ColCAT 3.6, containing 3521 base pairs of a1(I) collagen promoter DNA, fused to the CAT reporter gene, or an upstream deletion mutant of ColCAT 3.6 designated ColCAT 2.3. After 48 h, bFGF (0.1–10 nM) inhibited the incorporation of [3H]proline into collagenase-digestible protein (CDP). Indomethacin did not alter the inhibitory effect of bFGF on CDP labeling. Aphidicolin, an inhibitor of DNA synthesis, did not block the inhibitory effect of bFGF on CDP. bFGF (1–10 nM) decreased a1(I) procollagen mRNA levels, with maximal inhibition, nearly 99% of control, caused by 10 nM bFGF. After 48 h, bFGF (1 nm) reduced a1(I) procollagen gene transcription by about 92%. ColCAT 3.6 activity was inhibited with 0.1–10 nM bFGF and was maximally repressed by about 85% with 10 nM bFGF. In contrast, bFGF (1 and 10 nM) caused a stimulation of ColCAT 2.3 activity. These data show that bFGF inhibits collagen synthesis by a transcriptional mechanism and the a1(I) collagen promoter contains DNA sequences which mediate bFGF inhibition of type I collagen gene expression in bone.

Type I collagen, the product of the a1(I) and a2(I) genes, is the most abundant protein in bone. Expression of the type I collagen genes is controlled at multiple levels including developmental regulation (1, 2), tissue-specific expression (3, 4), inducible expression by agents such as transforming growth factor β (5) or repression by 1,25-(OH)2D3 (6). bFGF is a 16.5-kDa heparin binding growth factor which influences collagen gene transcription, and three 5' deletion mutants were fused to the chloramphenicol acetyltransferase (CAT) reporter gene (25). The expression of these transgenes in stably transfected osteoblastic cell lines revealed that the upstream promoter sequence between –3521 hp and –2985 bp contains one or more stimulatory elements which are preferentially active in osteoblasts (25). In the present study, we used nuclear run-on assays to demonstrate transcriptional inhibition of the a1(I) collagen gene by bFGF in osteoblastic MC3T3-E1 cells. Moreover, we have utilized the osteoblastic cell line MC3T3-E1 stably transfected with ColCAT 3.6 and its deletion constructs to begin to map DNA sequences within the rat a1(I) collagen promoter which mediate bFGF inhibition.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human bFGF was generously provided by Dr. John Fiddes, California Biotechnology, Inc. (Mountain View, CA). Guanidinium isothiocyanate, DNase, proteinase K, and agarose were obtained from Bethesda Research Laboratories. [3H]Proline (15 Ci/mmol) was obtained from Amersham. [methyl-3H]Thymidine (76.7 Ci/mmol), [3P]dGTP (3000 Ci/mmol) and [3P]UTP (3000 Ci/mmol) were obtained from Du Pont-New England Nuclear. Tissue culture dishes were purchased from Costar (Cambridge, MA). The assay for lactic dehydrogenase was performed utilizing a kit (DG1340-UV) obtained from Sigma.

* This work was supported by Public Health Service Grants AR-38983, AR-29650, and AR-29983. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom all correspondence and requests for reprints should be addressed: Division of Endocrinology and Metabolism, Dept. of Medicine, The University of Connecticut Health Ctr., Farmington, CT 06030-1850.

§ The abbreviations used are: bFGF, basic fibroblast growth factor; CDP, collagenase-digestible protein; NCP, noncollagen protein; PCS, percentage of the total protein represented by collagen; APC, aminopropylcollidine; TdR, thymidine; CAT, chloramphenicol acetyltransferase; PGE2, prostaglandin E2; TNFα, tumor necrosis factor α.
Cell Cultures—Immortalized osteoblast-like MC3T3-E1 cells were grown to confluence in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum (FCS), penicillin (100 µg/ml), and streptomycin (50 µg/ml). In addition, G418 (400 µg/ml) was added to cultures of transfected cells MC3T3-E1. Cells were plated at various densities, and experiments were begun 6–7 days later when the cells were confluent. Ascorbic acid (50 µg/ml) was added at the start of all experiments.

DNA Synthesis—To assess DNA synthesis, cells were treated with effectors for 24 h and incubated with 5 µCi/ml [3H]thymidine during the last 2 h of the culture period. At the end of the incubation, the medium was discarded, and cells were rinsed in cold phosphate-buffered saline and extracted with 1.0 ml of 10% trichloroacetic acid. Acid-extractable [3H]Thymidine was determined by measuring the radioactivity in the trichloroacetic acid washes. Then, 1 ml of 0.5 M NaOH was added to each well. Cells were scraped from the dishes, put into glass tubes, and incubated overnight at 4°C. An aliquot of each cell digest was counted to quantitate thymidine incorporation into DNA.

Collagen and Noncollagen Protein Synthesis—Protein synthesis in cells of different cell cultures was assessed by measuring the amount of [3H]proline incorporated into collagenase-digestible protein (CDP) and noncollagen protein (NCP) during the last 4 h of culture (29). Cells were labeled with 5 µCi of [3H]proline. Medium was removed and cells were scraped into extraction buffer (1 M NaCl, 1 mM N-ethylmaleimide, 0.2 M phenylmethylsulfonyl fluoride, 2.75 mM EDTA) and then recombined with medium. Protein was precipitated with 15% trichloroacetic acid, washed exhaustively with 10% trichloroacetic acid, and dissolved in 0.5 M NaOH. Aliquots of the cell homogenates were digested with bacterial collagenase to determine CDP and NCP labeling (27). The percentage of the total protein represented by collagen (PCP) was determined after correcting for the relative abundance of proline residues in collagen compared to noncollagen protein (29).

In order to determine whether the inhibitory effect of bFGF on collagen synthesis was due to its effects on cell replication, cells were precultured for 4 h with or without aphidicolin (APC) at 30 µM, an inhibitor of DNA synthesis. Then bFGF was added for 24 or 48 h. Collagen and noncollagen protein synthesis and TdR incorporation into DNA were determined as described above.

Steady-State Levels of mRNA—Total RNA was extracted from cells using the acid guanidium thiocyanate procedure of Chomczynski and Sacchi (29). For Northern analysis, 10-µg samples of total RNA were fractionated on a 1% agarose/1 M formaldehyde gel and then transferred onto nylon filters using positive pressure. RNA was immobilized by UV treatment (30). Filters were hybridized with a [32P]dUTP-labeled α1(I) procollagen cDNA probe (31). Northern blots were visualized by autoradiography and quantitated by densitometry. For normalization, filters were stripped and rehybridized with a radiolabeled β-actin cDNA probe, kindly provided by Dr. Don Cleveland (32) or glyceraldehyde-3-phosphate dehydrogenase cDNA probe (33).

Nuclear Run-on Assay—Cells were rinsed with phosphate-buffered saline, scraped from dishes, and harvested by centrifugation. Following suspension in homogenization in a reticulocyte standard buffer, nuclei were isolated and collected by centrifugation. Transcription was carried out for 20 min in a 200 µl reaction mixture containing nuclei, 50 mM Tris-HCl, pH 7.5, 100 mM ammonium sulfate, 3.75 mM KCl, 2.2 mM diithiothreitol, 1.8 mM manganese chloride, 2 µl of RNasin, 300 µM each of ATP, CTP, and GTP and 100 µCi of [32P]UTP. Then 10 µg/ml carrier yeast tRNA was added followed by 10 µg/ml RNase-free DNase and 100 µg/ml protease K. RNA was then extracted with phenol and chloroform and the aqueous phase precipitated with ethanol. After centrifugation the pellet was washed with 80% ethanol, dissolved in guanidium thiocyanate, and precipitated again with isopropanol. The RNA was harvested by centrifugation, resuspended in sterile water, and heated at 65°C for 10 min. RNA was hybridized to filter-immobilized plasmids containing appropriate cDNAs (6). Hybridized radioactivity was visualized by autoradiography and quantitated by liquid scintillation counting of the eluted radioactivity. Transcription rates were expressed as the ratio of radioactivity bound to specific cDNA probes minus radioactivity bound to pUC18 vector.

Transfection of DNA-MC3T3-E1 cells were plated at 4 × 10^4/cm^2 in 100-mm tissue culture dishes in Dulbecco's modified Eagle's medium containing 10% FCS. After 24 h, cells were cotransfected with 5 µg of an α1(I) collagen-CAT DNA construct and pSV-2+ (25) in a 1:1 ratio by the calcium phosphate precipitation method (34). After 6 h, the cells were shocked for 3 min with 10% glycerol, rinsed with phosphate-buffered saline, and then cultured in Dulbecco's modified Eagle's medium with 10% FCS and with 400 µg/ml of G418. Three to five weeks later resistant colonies were pooled, expanded, and assayed for CAT activity and collagen synthesis. Stably transfected low passage cells were stored frozen in liquid nitrogen.

Assay of CAT Activity—CAT activity in cell extracts was determined by a modification of a fluor diffusion assay (35,36). Cells were scraped into phosphate-buffered saline, resuspended in 0.25 M Tris-HCl, pH 7.8, and lysed by three freeze-thaw cycles. The extracts were heated at 65°C for 15 min and centrifuged to remove precipitated proteins. An aliquot of each cell extract was mixed with 0.1 µCi of [3H]acetyl-CoA, 1 mM chloramphenicol, and 0.1 M Tris-HCl, pH 7.8. The reaction mixture was overlayed with 5 ml of organic scintillation fluid. Vials were incubated at 37°C for up to 6 h and counted for 0.2 min every hour. After subtraction of a no enzyme background vial, linear regression analysis was performed. CAT activity is expressed as cpm/h/µg protein.

RESULTS

Immortalized murine MC3T3-E1 cells have an osteoblastic phenotype and have been shown to differentiate and produce a mineralized matrix in vitro (36). In MC3T3-E1 cells, 48 h of treatment with 0.1–10 nM recombinant human bFGF caused a concentration-dependent inhibition of [3H]proline incorporation into CDP which was maximally reduced by about 96% (Fig. 1). bFGF inhibited NCP labeling at a concentration of 1 nM or greater showing that other genes are inhibited by the effects of bFGF on collagen synthesis.
affected. However, when the data were normalized, there was a potent inhibitory effect of bFGF on collagen synthesis. bFGF over a similar concentration range also reduced collagen synthesis expressed as a percentage of total protein synthesis. Some of the biological responses of FGFs have been reported to be mediated by prostaglandin E₂ (PGE₂) production (19) and high concentrations of PGE₂ was previously reported to reduce type I collagen synthesis in bone (37). Thus, MC3T3-E1 cells were preincubated for 2 h with indomethacin, an inhibitor of PGE₂ production, prior to the addition of 1 nM bFGF for an additional 48 h. Indomethacin had no effect on basal CDP labeling, nor did it alter the inhibitory effect of bFGF on CDP labeling (Table I).

As shown in Tables I and II, bFGF at 1 and 10 nM significantly increased DNA content after 48 h. We tested whether the inhibition of collagen synthesis by bFGF was linked to its stimulation of cell replication. At 24 h, 30 µM APC, a DNA synthesis inhibitor, reduced TdR incorporation into DNA by greater than 90%. bFGF at 1 nM stimulated TdR by 621%, and this effect was blocked by 30 µM APC. Although APC alone inhibited CDP, the inhibitory effect of bFGF on CDP labeling was maintained in the presence of APC (Fig. 2).

The decrease in collagen synthesis by bFGF was accompanied by a decrease in steady state levels of α1(I) procollagen mRNA. Densitometric analysis of Northern blots revealed that a 48-h treatment with bFGF at 0.1, 1, and 10 nM decreased steady-state α1(I) procollagen mRNA levels by 68, 98, and 99%, respectively (Fig. 3). Similar results were obtained in two additional experiments (data not shown). Since high concentrations of bFGF also reduced β-actin mRNA the filters were stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase to normalize the data. There was little effect of bFGF on glyceraldehyde-3-phosphate dehydrogenase mRNA levels. The reduction in α1(I) procollagen mRNA was observed as early as 6 h and was maximal at 24 h (Fig. 4).

To determine whether bFGF acts at the level of type I
Regulation of Collagen Gene Expression by bFGF

FIG. 4. Time course for the effect of 10 nM bFGF on α1(I) procollagen mRNA levels in MC3T3-E1 cells. The data from three experiments were pooled.

FIG. 5. Effect of 1 nM bFGF on transcription of α1(I) procollagen gene in isolated nuclei from MC3T3-E1 cells. MC3T3-E1 were treated with bFGF for 48 h. The α1(I)/actin mRNA ratio mean and S.E. for control cultures was 3.73 ± 0.38 and for bFGF-treated cultures was 0.24 ± 0.14; p < 0.01. Transcription rates of the α1(I) procollagen gene were determined as described under “Experimental Procedures.”

collagen gene transcription, the rate of procollagen mRNA synthesis was measured in isolated nuclei from control and treated MC3T3-E1 cells. After 48 h of treatment, bFGF at 1 nM reduced α1(I) procollagen gene transcription by as much as 92% with a small reduction in transcription of the β-actin gene (Fig. 5).

The effects of bFGF on the α1(I) collagen promoter utilizing MC3T3-E1 cells stably transfected with either ColCAT 3.6 or ColCAT 2.3 were determined. Treatment of cells containing ColCAT 3.6 for 24 h with 1 nM bFGF reduced CDP and CAT activity by 90 and 64%, respectively. After a 48-h treatment, bFGF decreased CDP labeling and ColCAT 3.6 activity in a dose-dependent manner with bFGF at 10 nM reducing CDP by 97% and CAT activity by 83% (Table IIA). In five separate experiments bFGF at 10 nM reduced CDP by 91 ± 2% (range 83–98%), while ColCAT 3.6 activity was reduced by 71 ± 3% (range 56–86%). In contrast to its inhibitory effects on ColCAT 3.6 activity, bFGF at 1 and 10 nM actually increased CAT activity in MC3T3-E1 cells stably transfected with ColCAT 2.3 (Table IIB) even though the inhibitory effect of bFGF on CDP labeling was maintained.

Because previous studies have shown that MC3T3-E1 express the osteoblast phenotype 7–9 days after plating (38), we examined the effect of bFGF on CDP and ColCAT 3.6 activity in MC3T3-E1 cells plated at 5,000, 15,000, or 45,000 cells/cm². After 7 days of growth, cells were treated with bFGF for 48 h. Basal CAT activity was highest in transfected cells plated at high density. bFGF at 1 and 10 nM markedly inhibited CDP labeling and CAT activity in cells plated at 5,000 or 45,000 cells/cm² (Fig. 6). Similarly, Northern blot analysis revealed that high concentrations of bFGF decreased the endogenous α1(I) procollagen mRNA in cells plated at low or high density (Fig. 7). In subconfluent MC3T3-E1 cells, bFGF reduced CDP labeling and CAT activity after 48 h of treatment (data not shown).

To determine whether endogenous factors present in serum are necessary for mediating the effects of bFGF on collagen gene expression, MC3T3-E1 cells containing ColCAT 3.6 cells were plated at high density grown to confluence, serum deprived for 24 h and then treated with bFGF at 1 nM. Under these conditions, bFGF increased TdR incorporation into DNA by 393% and inhibited CDP by 81% and CAT activity by 64% (Table III). Therefore, bFGF can decrease collagen synthesis and CAT activity and stimulate DNA synthesis in the absence of serum factors.

FIG. 6. Dose response experiment for the effect of bFGF on CDP and CAT activity in MC3T3-E1 ColCAT 3.6 plated at different cell densities, grown for 7 days and treated for 48 h in the presence of 2% FCS. * different from control (p < 0.01); † different from 5000 cells/cm² (p < 0.01).
DISCUSSION

The results of this study show that bFGF decreases collagen synthesis and levels of α(I) procollagen mRNA in osteoblastic MC3T3-E1 cells. These changes are accompanied by a decrease in α(I) procollagen gene transcription indicating that bFGF acts on the α(I) procollagen gene by a transcriptional mechanism. Moreover, our data indicate that bFGF represses the activity of an α(I) collagen promoter suggesting that there are cis elements between −3.5 and −2.3 which are negatively regulated by bFGF. In multiple experiments the inhibitory effect of bFGF on collagen synthesis was greater than its effect on ColCAT 3.6 activity. There could be additional effects of bFGF on DNA sequences not represented in ColCAT 3.6. We cannot, however, rule out post-transcriptional effects of bFGF on collagen synthesis. In regard to other collagen genes, bFGF has been shown to decrease type II procollagen mRNA levels (39) and the activity of a type II collagen promoter (40) in chondrocytes.

bFGF is a potent mitogen for MC3T3-E1 cells, increasing the incorporation of radiolabeled thymidine and DNA content of cultures. We do not believe the potent inhibitory effect of bFGF on collagen synthesis was due to cell toxicity, since the highest concentrations of bFGF increased DNA content and increased cell number. Morphological examination revealed that there was an increase in the number of cells. In addition, assays for lactic dehydrogenase enzyme in the medium, a marker of cell death, showed no significant difference between control and treated cells. Moreover, the effect of bFGF on collagen synthesis was similar in the presence or absence of aphidicolin, an inhibitor of DNA synthesis, this inhibitory effect of bFGF on collagen synthesis and ColCAT 3.6 activity were observed in cells plated at high or low density and in subconfluent cultures, suggesting that these inhibitory effects of bFGF are independent of the plating density.

The mechanism(s) by which bFGF negatively regulates collagen gene transcription in MC3T3-E1 cells is unknown. Both protein kinase C-dependent and independent signal transduction pathways appear to mediate the biological effects of bFGF in other tissues. The mitogenic signaling pathway of bFGF appears to be mediated through protein kinase C with activation of c-fos and c-jun genes and synthesis of the nuclear proto-oncogene products c-Fos and c-Jun which binds to DNA elements containing AP-1 (14, 41). In contrast, some studies have suggested that the effect of bFGF on induction of plasminogen activator or inhibition of muscle cell differentiation is independent of protein kinase C activation (42). Recently, constitutive expression of the c-fos gene was shown to inhibit collagen synthesis in MC3T3-E1 cells (43). It is interesting to note that the marked degree of c-fos induced inhibition of collagen synthesis reported in the above study (43) is similar to that induced by bFGF in our present report. Whether the inhibitory effect of bFGF on collagen gene expression is mediated by induction of c-fos gene has not been determined.

In contrast to the high basal expression of ColCAT 2.3, the activity of ColCAT 2.3 was actually increased by bFGF. bFGF decreased ColCAT 3.6 activity about 5–6-fold and increased ColCAT 2.3 activity about 5-fold. bFGF decreased ColCAT 3.6 activity about 5–6-fold and increased ColCAT 2.3 activity about 15-fold. It is possible that deletion of the strong upstream basal enhancer (−3.5 to −2.3) reveals additional effects of bFGF on other regions of the promoter. It is possible that this stimulation of the low basal activity of ColCAT 2.3 represents the “unmasking” of a DNA element in the −2.3 to −1.7-kb region of the α(I) collagen promoter which is positively regulated by bFGF. The rat α(I) collagen promoter fragment isolated by Lichtler et al. (44) has a number of transcription factor elements including several putative AP-1 sites. It is possible that the AP-1 element at −2.25 kb may have a positive effect on collagen promoter activity.

PGE₂ has biphasic effects on collagen synthesis in organ culture and has been shown to decrease collagen gene transcription, collagen synthesis, and ColCAT 3.6 activity in osteoblastic cells (45). We therefore assessed the role of endogenous PGE₂ in mediating the effects of bFGF on the collagen gene. Since indomethacin did not abrogate the inhibitory effect of bFGF on collagen gene transcription, we conclude that the effects of bFGF on collagen synthesis and ColCAT 3.6 activity are not mediated by endogenous PGE₂.

In previous studies Katagari and co-workers (38) reported that the response to TGFβ treatment in MC3T3-E1 cells changed depending on their maturation stage. We therefore determined whether the response to bFGF similarly depended on the extent of cell maturation. In the present study, the inhibitory effect of bFGF on collagen synthesis and ColCAT 3.6 activity was seen in cells plated at high or low density and in subconfluent cultures, suggesting that these inhibitory effects of bFGF are independent of the plating density.
Recent studies have suggested that cytokines such as tumor necrosis factor α (TNFα) or transforming growth factor β can negatively regulate collagen synthesis in fibroblasts and chondrocytes (39, 46, 47). In the case of TNFα, this inhibition appears to be at the level of collagen gene transcription (46, 47). Since treatment of endothelial cells with TNFα increased bFGF mRNA levels within 3 h (48), it is interesting to speculate that the TNFα induced inhibitory effects on the type I collagen gene is mediated in part by endogenous bFGF.

In summary, these data show that bFGF regulates collagen gene expression at the transcriptional level and that there is an inhibitory locus (i) located between −3.5 and −2.3 kb within the a1(I) collagen promoter. Since bFGF is localized to bone matrix (17) and has recently been shown to increase bone resorption (20) and inhibit bone formation (22, 49), bFGF may affect bone remodeling by inhibiting this synthesis of type I collagen a major bone matrix protein.

REFERENCES