Antisense Inhibition of Basic Fibroblast Growth Factor Induces Apoptosis in Vascular Smooth Muscle Cells*

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Basic fibroblast growth factor (bFGF), a potent mitogen for many cell types, is expressed by vascular smooth muscle cells and plays a prominent role in the proliferative response to vascular injury. Basic FGF has also been implicated as a survival factor for a variety of quiescent or terminally differentiated cells. Autocrine mechanisms could potentially mediate both proliferation and cell survival. To probe such autocrine pathways, endogenous bFGF production was inhibited in cultured rat vascular smooth muscle cells by the expression of antisense bFGF RNA. Inhibition of endogenous bFGF production induced apoptosis in these cells independent of proliferation, and apoptosis could be prevented by exogenous bFGF but not serum or epidermal growth factor. The induction of apoptosis was associated with an inappropriate entry into S phase. These data demonstrate that interruption of autocrine bFGF signaling results in apoptosis of vascular smooth muscle cells, and that the mechanism involves disruption of normal cell cycle regulation.

EXPERIMENTAL PROCEDURES

Recombinant Adenoviruses—The Ad.ASbFGF virus was constructed by replacement of the β-galactosidase cDNA with a 1.1-kilobase rat bFGF cDNA containing the entire coding sequence plus 5′-untranslated region sequences (gift of Dr. Andrew Baird, Whittier Institute; GenBank accession number M22427) (26) in the pAd.CMVlacZ shuttle vector (gift of Dr. James Wilson, University of Pennsylvania). The resulting shuttle vector pAd.CMV.ASbFGF was cotransfected with the Ad5/RSV/GL2 encoding firefly luciferase (gift of Dr. Brent French, University of Pennsylvania) (27) or the lacZ shuttle vector pAd.CMV.lacZ (gift of Dr. James Wilson, University of Pennsylvania) (28). This procedure was transduced into human embryonal kidney 293 cells (CRL-1573, American Type Culture Collection, Bethesda MD), generating the recombinant adenovirus Ad.ASbFGF by homologous recombination (28). Either Ad.CMVlacZ encoding bacterial β-galactosidase (gift of Dr. James Wilson, University of Pennsylvania) (27) or Ad5RSV/GL2 encoding firefly luciferase (gift of Dr. Brent French, Baylor School of Medicine) (29), were used as control viruses in these 

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1 The abbreviations used are: bFGF, basic fibroblast growth factor; FGF, fibroblast growth factor; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FBS, fetal bovine serum.
and subsequent experiments.

Recombinant adenovirus was identified by PCR amplification of putative recombinant plaques using one vector-specific and one insert-specific primer. The vector-specific primer (5'-AGACATGATAAGACTCAT-3') corresponds to a region of the shuttle vector upstream of the insert cloning site but contains no adenoviral genomic sequence. The insert-specific primer (5'-TCGCTCCGAGTTG AGGTA-3') corresponds to codons 37–42 of the 18-kDa rat bFGF coding sequence. The recombinant virus was twice plaque-puriﬁed, and expression of the antisense transcript was conﬁrmed by reverse transcription-PCR (RT-PCR). Total cellular RNA (1 µg) isolated from uninfected smooth muscle cells or cells infected with either Ad.As.bFGF or Ad.SRV/GL was heat-denatured (65°C) and reverse-transcribed using a random hexamer sequence-speciﬁc primer. This primer contains 18 bases (5'-ACTTGCTCCTGCCGATCGT-3') of bFGF sequence complementary to the antisense strand (corresponding to codons 7–12 of 18-kDa rat bFGF) at the 3’ end, as well as a 30-base random sequence at the 5’ end (5'-CTTATACGGATCCTGCGATGAGGACTAGT-3'). The 5’ end random sequence tag permits subsequent PCR ampliﬁcation with a primer corresponding to this tag sequence only, thus rendering the overall ampliﬁcation both strand- and RNA-speciﬁc (30). The 5’ primer used for the PCR ampliﬁcation (5'-GCACACACTCCCTCAGGAC-3') corresponds to codons 71–78 of 18-kDa rat bFGF. As controls, each of the cellular RNAs was also reverse-transcribed using random hexamer primers (1 pmol), and these cDNA products were subsequently ampliﬁed using the above-described PCR primer and reverse transcriptase. Ampliﬁed products were sequenced using primers (1 pmol), and these cDNA products were subsequently ampliﬁed in vitro using cDNA templates and bacteriophage T7 RNA polymerase as well as a myeloblastosis virus reverse transcriptase (Boehringer Mannheim) or 50 ng/ml human EGF (Upstate Biotechnology) was added diurnally to the cultures, cells were pulse-labeled with 10 µCi/ml [3H]Thymidine for 3 h. At the end of the labeling period, cells were collected with trypsin and subjected to hypotonic lysis (31). For the detection of oligonucleosomal fragmentation, cells were collected with trypsin and subjected to hypotonic lysis in buffer containing Triton X-100, and a low molecular weight DNA fraction was isolated as described (33). The low molecular weight fraction from equal numbers of cells was separated by electrophoresis through agarose, stained with ethidium bromide, and visualized by transillumination with UV light using an Eagle Eye apparatus (Strategene, La Jolla, CA). Total cellular DNA isolated from γ-irradian exposure of rat embryo fibroblasts exposed to 10 GY of γ-ray (gift of E. Bernhard) was used as a positive control for oligonucleosomal fragmentation.

Cell Cycle Phase Determination and BrdU Labeling—
For flow cytometric analysis of cell cycle phase distribution, cells were infected with recombinant adenoviruses and harvested with trypsin, and nuclei were prepared and stained with propidium iodide as described (34). Flow cytometric analysis was performed using a FACScan cytometer (Becton-Dickinson, San Jose, CA) and analyzed using ModFit LT curve-fitting software (Verity Software, Topsham, ME) running on a Macintosh computer (Apple Computer, CA). For tritiated thymidine labeling, sham-or adenovirus-infected cells were labeled 2 days following infection for 12 or 18 h in serum-free medium containing 2–3 µCi/ml [methyl-3H]thymidine (Amersham Corp.), fixed in trichloroacetic acid, solubilized in dilute NaOH, and collected by filtration, and incorporation was determined by scintillation counting (Beckman Instruments, Columbia, MD).

RESULTS

Antisense Inhibition of Endogenous bFGF Triggers Smooth Muscle Cell Death—Expression of an antisense bFGF RNA (Fig. 1A) by a recombinant adenovirus (Ad.As.bFGF) in cultured smooth muscle cells markedly reduced cellular bFGF content (Fig. 1B). To test the hypothesis that inhibition of endogenous bFGF expression reduces either smooth muscle cell proliferation or survival, cell number was monitored following Ad.As.bFGF infection (Fig. 2). The expected accumulation of smooth muscle cells cultured in 10% FBS was inhibited by Ad.As.bFGF in a dose-dependent fashion (panel A) and could be prevented by exogenous bFGF (panel B). In order to distinguish between an inhibition of proliferation and promotion of cell death, quiescent smooth muscle cells were infected with the antisense bFGF virus and maintained in low (0.5%) serum. The number of antisense-infected cells decreased in a dose-dependent fashion (panel C), indicating cell death, which was subsequently identified as apoptosis by accepted morphologic and biochemical criteria.

Criteria for Apoptosis in Cells Infected with the Antisense bFGF Adenovirus—Cells infected with the antisense bFGF virus appeared normal for the first 24 h, but thereafter alter-
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Rat aortic smooth muscle cells were sham-infected or infected with either Ad.ASbFGF or Ad5/RSV/GL2 recombinant adenoviruses at a multiplicity of infection (MOI) of 1000. RT-PCR analysis (A) was performed on total RNA isolated from cells infected with either Ad.ASbFGF (lane 2, AS) or Ad5/RSV/GL2 (lane 3, Luc) and from uninfected cells (lane 4, Un). Controls included a synthetic AS-bFGF transcript generated from a cDNA template in vitro (lane 5, +C) and a no template control (lane 1, -C). Each of the cellular RNAs was also analyzed for a control mRNA transcript, GAPDH, using a synthetic GAPDH transcript containing a short deletion (GAPDHdel) as a positive control. This experiment was repeated at least five times using independent RNA samples and alternative primer sets with similar results. Basic FGF cell content was determined by Western (immunoblot) analysis (B). Both uninfected cells (lane 1) and cells infected with Ad5/RSV/GL2 (lane 2) contain equivalent amounts of all three isoforms of rat bFGF, whereas Ad.ASbFGF-infected cells (lane 3) contain much less immunoreactive bFGF. This analysis was repeated three times with similar results.

Antisense inhibition of endogenous bFGF synthesis provokes cell death in either high or low serum, which can be prevented by exogenous bFGF. Antisense inhibition of bFGF expression reduces the accumulation of cells cultured in 10% FBS (A). Cells were infected with Ad.CMVlacZ at an MOI = 1000 (open squares), or Ad.ASbFGF at an MOI = 50 (filled diamonds), 100 (filled triangles), or 1000 (filled circles) and maintained in 10% FBS. Cells were counted daily starting on the day after infection; individual data points in this and each of the experiments in this figure represent the mean ± S.D. of triplicate wells, and each experiment was repeated at least three times. For the bFGF rescue experiment (B), cells were infected with Ad.CMVlacZ or Ad.ASbFGF at an MOI = 100; bFGF was added daily starting 1 day following infection, and cell counts were monitored. Cells infected with Ad.CMVlacZ and cultured in the absence (open squares), or presence (open circles) of bFGF were compared with cells infected with Ad.ASbFGF in the absence (filled squares) or presence (filled circles) of bFGF. To distinguish between inhibition of proliferation and promotion of cell death, cells were cultured in 0.5% serum to maintain quiescence both prior to and following infection with recombinant adenoviruses (C). Cells were infected with either Ad5/RSV/GL2 at an MOI = 1000 (open squares) or with Ad.ASbFGF at an MOI = 100 (filled triangles) or 1000 (filled circles), and cell counts were monitored daily.

Epidermal Growth Factor Does Not Prevent Apoptosis Triggered by Ad.ASbFGF

Second type of apoptotic mechanism involving inappropriate S phase entry. To distinguish between these two possibilities, the ability of a different growth factor to prevent apoptosis following infection with Ad.ASbFGF was assessed, and the degree of S phase entry was estimated by flow cytometry, rates of DNA synthesis, and BrdU labeling of apoptotic nuclei in Ad.ASbFGF-infected cells.
the reduced accumulation of cells over time does not distinguish between the prevention of cell death and the stimulation of proliferation in the surviving cells. In order to make this distinction, the extent of apoptosis in the presence or absence of growth factors was assessed by the percentage of apoptotic nuclei visualized by propidium iodide staining. In cells cultured in 0.5% FBS in the absence of growth factors and infected at an MOI of 1000 with either Ad.ASbFGF or control virus, the percentage of apoptotic nuclei in control virus-infected cells was 0.5 ± 0.5% (mean ± S.D. of triplicate samples) compared with 18.9 ± 4.4% in Ad.ASbFGF-infected cells, which was also associated with a 20% decrease in cell number. In Ad.ASbFGF-infected cells treated with bFGF, the percentage of apoptotic nuclei was only 8 ± 1.1% compared with 15.5 ± 4.7% in cells treated with EGF, despite a similar (2-fold) increase in cell number stimulated by either growth factor in both control virus- or Ad.ASbFGF-infected cells. These data show that despite a similar stimulation of proliferation in non-apoptotic cells, bFGF but not EGF was able to reduce the proportion of apoptotic cells. This result suggests that apoptosis triggered by inhibition of autocrine bFGF synthesis does not correspond to a growth factor withdrawal model of apoptosis or that it involves signaling pathways that are not shared by bFGF and EGF. An alternative hypothesis, that apoptosis triggered by inhibition of autocrine bFGF synthesis involves disruption of normal cell cycle regulation, was tested by examining several complementary measures of cell cycle activity.

Apoptosis Induced by Ad.ASbFGF Involves Inappropriate S Phase Entry—Analysis of cell cycle activity in Ad.ASbFGF-versus control virus-infected smooth muscle cells suggested that an inappropriate entry into S phase participates in the mechanism of apoptosis due to inhibition of autocrine bFGF synthesis. By flow cytometric analysis, Ad.ASbFGF-infected cells displayed a marked increase in the proportion of cells in S phase at the expense of the G0/G1 population (Fig. 5), and a time course experiment demonstrated an abrupt transition from a normal cell cycle phase distribution to one characterized by an increase in S phase between 16 and 20 h after infection (data not shown). These findings were confirmed by measuring rates of DNA synthesis by tritiated thymidine labeling (Fig. 6), which were greater in Ad.ASbFGF-infected versus control cells. The magnitude of the difference in thymidine labeling appeared to be dose-related (Fig. 6A), and at the higher MOI thymidine incorporation was about 2-fold the value in control cells. This result could be interpreted to mean that twice as
many Ad.ASbFGF-infected cells were incorporating thymidine as control cells, an interpretation consistent with the flow cytometry results. This suggests that the excess DNA synthesis in the Ad.ASbFGF-infected cells was due to apoptosis, and this was made more apparent by thymidine labeling in quiescent cells (Fig. 6B). Under these circumstances, the thymidine incorporation in the control cells is very low, and the difference in labeling is apparently due to DNA synthesis in the proportion of cells undergoing apoptosis. This conclusion was supported by BrdU pulse labeling and immunofluorescence imaging of cells that had been infected with either Ad.ASbFGF or control viruses (Fig. 7), which showed that approximately 20% of cells were BrdU-labeled and that about half of these labeled cells had apoptotic (condensed or fragmented) nuclei, demonstrating that the increased DNA synthesis was associated with apoptosis and was not simply a compensatory response of the surviving cells. These data demonstrate that apoptosis induced by inhibition of bFGF synthesis involves an inappropriate entry into S phase, similar to other models of apoptosis involving disrupted cell cycle regulation, and suggests that autocrine FGF signaling participates in cell cycle regulation as part of its survival function.

DISCUSSION

This study demonstrates that inhibition of endogenous basic FGF synthesis using an antisense strategy triggers apoptosis in cultured vascular smooth muscle cells, supporting an essential survival function for autocrine FGF signaling in this cell type. Prior studies implicating fibroblast growth factors as survival factors in mesodermal and neuroectodermal cells (14, 16, 17, 19, 20, 31, 44) suggest that this survival function is related to prevention of apoptosis (19, 20, 44) but do not distinguish between mitogenic and survival-promoting activities.

Overexpression of FGF can lead to a transformed phenotype (45–50), supporting the idea that autocrine FGF signaling can drive proliferation, but interactions between FGFs and transmembrane FGF receptors may be required for FGF-stimulated mitogenesis (45–49). On the other hand, selective overexpression of the high molecular weight isoforms of bFGF can either promote or inhibit proliferation without evidence of receptor interaction (50, 51). None of these studies establish that autocrine FGF signaling is either necessary or sufficient for proliferation in cells capable of such autocrine loops. The mechanism of apoptosis in the present study appears to be independent of proliferation, as it occurs in cells cultured in either high (10%) or low (0.5%) serum. The ability of exogenous bFGF to prevent apoptosis may be related to activation of receptor-mediated signal transduction pathways or to internalization of ligand per se, although the inability of epidermal growth factor to block this mechanism of apoptosis suggests that receptor-mediated signaling may be less important. Thus the survival function of endogenous bFGF in smooth muscle cells appears to involve signal transduction pathways that may be distinct from those mediating mitogenesis. Experiments in transfected skeletal myoblasts (52) and fibroblasts (51) support important differences between FGF signaling pathways controlling mitogenesis and other cell functions.

The data presented establish that the pathway of apoptosis triggered by inhibition of autocrine FGF signaling involves an inappropriate entry into S phase. The possibility that DNA
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repair mechanisms contribute to the observed increase in total DNA synthesis has not been excluded, but the cell cycle phase analysis suggests that S phase entry is the predominant, if not exclusive, mechanism responsible. In this aspect it resembles other models of apoptosis that are due to disruption of normal cell cycle regulation. Unregulated expression of c-myc (39, 40) or E2F (41, 42) induces an inappropriate S phase entry and triggers apoptosis in the absence of serum. These pathways of apoptosis can be understood, at least in the case of E2F, by alteration in the regulatory balance between E2F and the retinoblastoma gene product, normally regulated by cyclins and cyclin-dependent kinase inhibitors. It is possible that expression of adenoviral genes in conjunction with the antisense bFGF transcript could be contributing to the observed phenotype. The adenoviral protein E1A interacts with cell cycle regulators such as the retinoblastoma protein and is a recognized inducer of apoptosis (22, 53). Although the recombinant viruses used in this study are specifically E1-deleted, contamination with E1-expressing virus, although highly unlikely, has not been rigorously excluded. Although a direct relationship between FGF signaling and the activities of cell cycle regulatory proteins has not been demonstrated, the present study provides more direct evidence of the ability of autocrine FGF signaling to influence cell cycle regulation than has previously been reported.

Antisense inhibition of endogenous bFGF expression leads to an interruption of autocrine FGF signaling that appears to alter cell cycle regulation, resulting in apoptosis of vascular smooth muscle cells. These observations provide a mechanism that could explain the survival function of FGFs expressed in quiescent or terminally differentiated cells. The expression of FGFs is widely detectable in mesodermal and neuroectodermal lineages of adult organisms, and autocrine FGF signaling may well play a role in the cellular homeostasis and survival of these cell types. The present work provides insights into the autocrine functions of FGF and the signaling pathways whereby endogenous FGF prevents apoptosis are currently being explored.

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