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# Distinct Patterns of Expression of Fibroblast Growth Factors and Their Receptors in Human Atheroma and Nonatherosclerotic Arteries

## Association of Acidic FGF with Plaque Microvessels and Macrophages

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### Abstract

Because fibroblast growth factors (FGFs) modulate important functions of endothelial cells (EC) and smooth muscle cells (SMC), we studied FGF expression in human vascular cells and control or atherosclerotic arteries. All cells and arteries contained acidic (a) FGF and basic (b) FGF mRNA. Northern analysis detected aFGF mRNA only in one of five control arteries but in all five atheroma tested, while levels of bFGF mRNA did not differ among control ( $n = 3$ ) vs. plaque specimens ( $n = 6$ ). Immunolocalization revealed abundant bFGF protein in control vessels ( $n = 10$ ), but little in plaques ( $n = 14$ ). In contrast, atheroma ( $n = 14$ ), but not control arteries ( $n = 10$ ), consistently exhibited immunoreactive aFGF, notably in neovascularized and macrophage-rich regions of plaque. Because macrophages colocalized with aFGF, we tested human monocytoid THP-1 cells and demonstrated accumulation of aFGF mRNA during PMA-induced differentiation. We also examined the expression of mRNA encoding FGF receptors (FGFRs). All cells and arteries contained FGFR-1 mRNA. Only SMC and control vessels had FGFR-2 mRNA, while EC and some arteries contained FGFR-4 mRNA. The relative lack of bFGF in plaques vs. normal arteries suggests that this growth factor may not contribute to cell proliferation in advanced atherosclerosis. However, aFGF produced by plaque macrophages may stimulate the growth of microvessels during human atherogenesis. (*J. Clin. Invest.* 1993. 92:2408–2418.)  
Key words: basic fibroblast growth factor • atherosclerosis • macrophages • neovascularization • angiogenesis

### Introduction

Fibroblast growth factors (FGFs) exert wide-ranging effects on various cell types (1–4). In vitro FGFs can stimulate proliferation, morphologic changes, and migration of vascular EC<sup>1</sup> and

SMC (1–4). In vivo these growth factors participate in development (1–4), vasculogenesis (5), angiogenesis (6, 7), and wound healing (6). Acidic FGF (aFGF) and basic FGF (bFGF), also known as FGF-1 and FGF-2, respectively (8), are the best characterized members of the FGF family. A complex superfamily of cell surface receptors with tyrosine-kinase activity mediates FGFs' effects on target cells (9, 10).

Recent studies support the involvement of aFGF and bFGF in vascular pathobiology. Animal experiments have established that these growth factors can foster re-endothelialization (11, 12), mediate SMC replication (12–14), and stimulate formation of vasa vasorum (14) in balloon-injured arteries. Although vascular EC and SMC can both produce and respond to aFGF and bFGF in vitro, information is incomplete regarding expression and localization of these growth factors and their receptors in either normal (15) or atherosclerotic (15, 16) human arteries. This study explored these issues.

We documented differential patterns of expression of FGF family members and of their receptors in cultured human EC and SMC and in atherosclerotic and control arteries. In particular, we found increased levels of aFGF mRNA in human atheroma compared to nonatherosclerotic arteries and association of aFGF protein with plaque microvessels and macrophages. In contrast, atheroma contained less immunoreactive bFGF protein than control arteries.

### Methods

**Cell culture.** Human umbilical vein EC and aortic SMC were grown as previously described (17). Human embryonal carcinoma cells (TERA-2), purchased from the American Type Culture Collection, Rockville, MD, were grown in medium 199 with 10% FCS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 0.25  $\mu$ g/ml amphotericin B (complete M199). Human skin fibroblasts (GM2037), purchased from the Human Genetic Mutant Cell Repository, Camden, NJ, were grown as previously described (18). Human monocytes were isolated from leukocyte-rich fractions collected after leukapheresis from healthy donors. Mononuclear cells were layered onto Ficoll hypaque (Pharmacia LKB Biotechnology, Piscataway, NJ) and separated by centrifugation (2,500 rpm, 25 min). The mononuclear cell band was washed twice with HBSS without Ca<sup>2+</sup> and Mg<sup>2+</sup>, resuspended in medium RPMI 1640 with L-glutamine, 10% FCS, 25 mM Hepes, and 1% penicillin-streptomycin (complete RPMI) and permitted to adhere to polystyrene flasks. After 2 h, nonadherent cells were removed by washing gently three times with HBSS and medium was replaced. Mono-

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1. **Abbreviations used in this paper:** aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; complete M199, medium 199 with 10% FCS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 0.25  $\mu$ g/ml amphotericin B; complete RPMI, medium RPMI 1640 with L-glutamine, 10% FCS, 25 mM Hepes, and 1% penicillin-streptomycin; EC, endothelial cells; FGFR, fibroblast growth factor receptor; GITC, guanidinium isothiocyanate; RT, reverse transcription; SMC, smooth muscle cells; TBS, tris-buffered saline.

Table I. Primers used for PCR Analysis

Gene	Sense	Antisense	bp	Ref
aFGF	GCTGCTGAGGCCATGGCTGAA	ACAGATCTCTTTAATCAGAAGAGACTG	489	40
bFGF	GGAGTGTGTGCTAACCGTTACCTGGCTATG	TCAGCTCTTAGCAGACATTGGAAGAAAAAG	243	41
FGF-3	ATGGGCCTAATCTGGCTGCTACTG	CAAACCTCGACTCGGCGCTGTAGT	349	42
FGF-4	GACTACCTGCTGGGCATCAAGCGG	GATGAACATGCCGGGGTACTTGTA	314	43
FGF-5	TCCAGCAGCAGACAGAGCAGCAGT	TGAGTTGGTATTTTTCCGAGGTGC	641	44
FGF-6	AGGCACCCGTGCCAACACACGCT	TGACAGTCATGATCGGGGACACCT	484	45
FGF-7	CTTTGCTCTACAGATCATGCTTTC	TTGCCATAGGAAGAAAGTGGGCTG	542	34
FGFR-1	AAGGACAAACCAACCGTGTGACC	CCCAAAGTCTGCTATCTTCATCAC	425	46
FGFR-2	CTGTGCCGAATGAAGAACACGACC	CCCAAAGTCTGCTATCTTCATCAC	743	47
FGFR-3	TGAGGACACAGGTGTGGACACAGG	TCTGCCGGATGCTGCCAAACTTGT	295	36
FGFR-4	CTCGCCGGCCTCGTGAGTCTAGAT	CCCAAAGTCTGCTATCTTCATCAC	560	37
Actin	ACCCTGAAGTACCCCAT	TAGAAGCATTGCGGGT	931	48

Primer pairs were designed to amplify selectively a specific fragment of the target sequence investigated. bp refers to the size of the PCR product expected and observed for each primer pair. Primer sequences are 5' to 3'.

cytes, identified by positive staining with EBM-11 antibody (1:4,000 dilution; Cat. no. M718; Dako Corp., Carpinteria, CA), were cultured and used at various time points after isolation. Cells of the human monocytoid leukemia cell line THP-1 (19), adapted to long-term culture, were passaged in complete RPMI at a concentration of  $2 \times 10^5$  cells/ml, but plated at higher density ( $6 \times 10^5$  cells/ml) for experimental use. All components used in tissue culture media were tested for endotoxin contamination using the chromogenic *Limulus* amoebocyte lysate assay (QCL 1,000; M.A. Bioproducts, Walkersville, MD). All medium constituents yielded a final concentration of endotoxin less than 40 pg/ml.

**Arterial tissue.** 14 carotid plaques were obtained at endarterectomy and processed within 2 h after surgery. Each specimen was rinsed twice in cold PBS to minimize blood residue, then a portion was snap-frozen in optimal cutting temperature tissue processing medium (OCT; Miles Diagnostics, Elkhart, IN) for immunohistochemical study and the remaining part was flash-frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  for RNA isolation. Although the study did not include heavily calcified plaques, all atherosclerotic samples studied contained focal calcifications at microscopic examination. 10 control human arteries (4 aortas, 1 coronary, 1 subclavian, and 4 carotid arteries) were obtained from the New England Organ Bank or the Department of Surgery and handled similarly. Control arteries had a grossly normal appearance. At microscopic examination, six of them exhibited a degree of intimal thickening within normal limits for human arteries.

**RNA isolation from cultured cells and tissue samples.** In short-term experiments human monocytes and THP-1 cells were stimulated with: PMA, 25 nM (cat. no. P-8139, Sigma Chemical Co., St. Louis, MO); palmitoyl L-alpha-lysophosphatidylcholine, 100  $\mu\text{M}$  (cat. no. L-5254, Sigma Chemical Co.); IFN-gamma, 500 U/ml (R&D Systems, Minneapolis, MN); LPS from *Escherichia coli* 055:B5, 10 ng/ml (cat. no. L-2880, Sigma Chemical Co.), and PDGF-BB, 10 ng/ml (Amgen Inc., Thousand Oaks, CA). Cells were harvested after 0, 2, 6, 10, 24, and 72 h of treatment. In a longer time course study, THP-1 cells were continuously stimulated with PMA, 100 nM for 4, 7, 14, and 17 d. Unstimulated control cells were harvested at day 0 and 17. During the course of long-term experiments culture medium, with or without addition of PMA, was replaced every other day. Total RNA from TERA-2 cells and human skin fibroblasts was prepared as described (17). SMC were growth-arrested for 48 h (20), stimulated for 6 h with PMA (25 nM), and extracted in GITC (21). Tissue samples were homogenized in GITC and total RNA extracted (21). RNA concentration was calculated from the absorbance at 260 nm. All RNA samples contained intact rRNA and lacked genomic DNA as evaluated by ethidium bromide staining of agarose-formaldehyde gels. Other RNA samples (hu-

man brain, lung, skeletal muscle) used as positive controls in reverse transcription (RT)-PCR reactions, were purchased from Clontech, Palo Alto, CA.

**Northern blot analysis.** Equivalent amounts of RNA (10 or 15  $\mu\text{g}$ , depending on the experiment) were separated on a 1.2% agarose gel containing 2.2 M formaldehyde. Gels were stained with ethidium bromide to verify loading of similar amounts of undegraded RNA. After

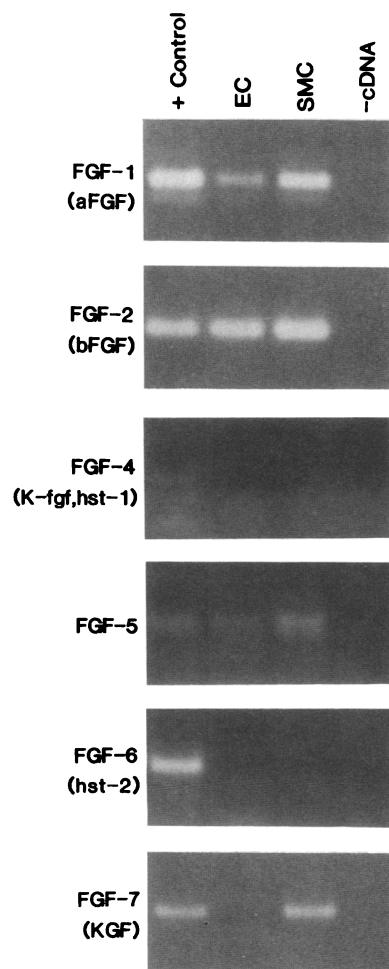
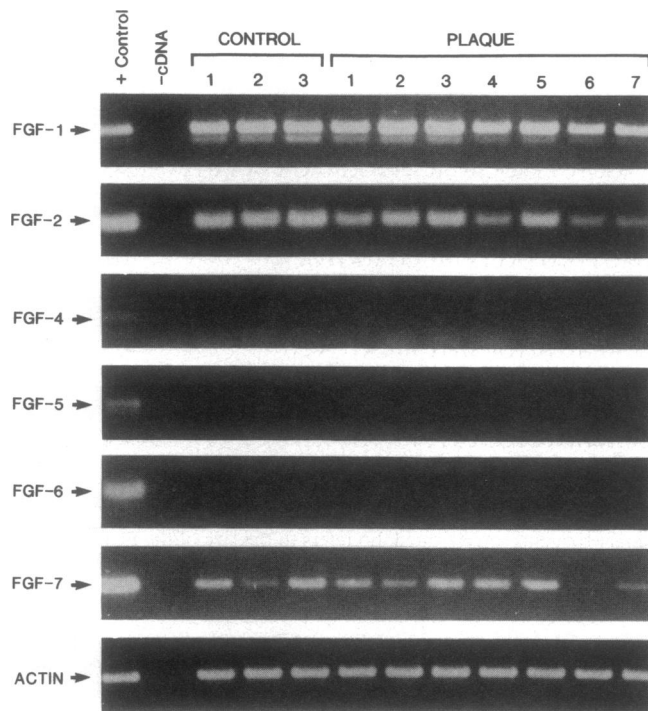


Figure 1. EC and SMC express aFGF, bFGF, and FGF-5 mRNA in vitro. SMC also express FGF-7 mRNA. This figure shows results of RT-PCR analysis at 35 cycles. We detected expression of aFGF, bFGF, and FGF-5 mRNA in human EC in vitro. Aortic SMC expressed aFGF, bFGF, FGF-5, and FGF-7 mRNA. Sources of positive control RNA were: human brain for aFGF and bFGF; TERA-2 cells for FGF-4; human skin fibroblasts for FGF-5 and FGF-7; and skeletal muscle for FGF-6.



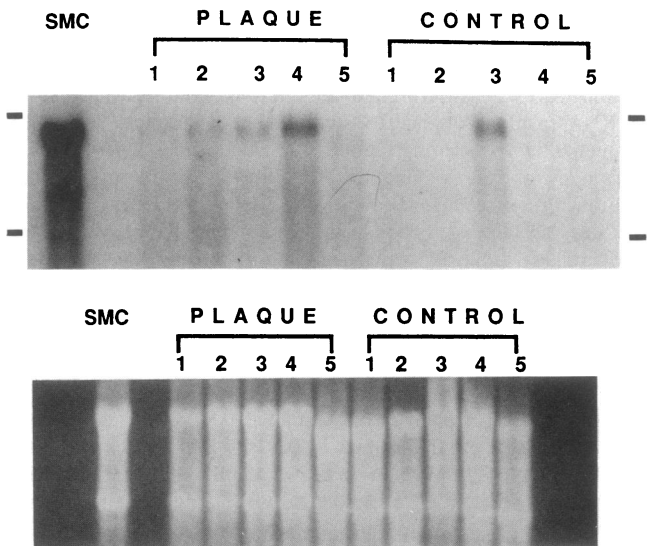
**Figure 2.** Control and atherosclerotic arteries express aFGF, bFGF, and FGF-7 mRNA. This figure shows results of RT-PCR analysis at 35 cycles. All control and atherosclerotic arteries contained aFGF mRNA; they also contained inconsistent levels of bFGF mRNA. Neither control nor atherosclerotic plaques contained FGF-5 mRNA, although this transcript was detected in cultured aortic SMC (Fig. 1). Both control and atherosclerotic arteries contained FGF-7 mRNA. Sources of positive control RNA were: human arterial SMC for aFGF, bFGF, FGF-5, and FGF-7; TERA-2 cells for FGF-4; and skeletal muscle for FGF-6. Identical volumes of the reaction mixture, but without addition of cDNA, served as negative controls. PCR reactions using beta-actin specific primers demonstrated that equal amounts of cDNA had been used and similarly amplified.

capillary transfer to nylon membranes (Hybond-N; Amersham Corp., Arlington Heights, IL), RNA was immobilized by short-wave UV irradiation (Stratalinker; Stratagene Corp., La Jolla, CA). Hybridization probes were labeled with [ $\alpha$ - $^{32}$ P]dCTP (New England Nuclear, Boston, MA) by random priming; free nucleotides were removed using a Sephadex G-50 column (Worthington Biochemical Corp., Freehold, NJ). Probes had specific activities of  $2\text{--}5 \times 10^8$  cpm/ $\mu$ g. The DNA probes were: (a) for human aFGF, a 2.2-kb EcoRI fragment of pDH15, purchased from American Type Culture Collection; (b) for bovine bFGF, a 1.4-kb EcoRI fragment of pJJ11 (gift of Dr. J. Abraham, Scios Nova Inc., Mountain View, CA); (c) for human FGFR-1, a 0.7-kb EcoRI/HindIII fragment of *flg* PCR-R (gift of Dr. M. Jaye, Rhone-Poulenc Rorer Pharmaceuticals, Collegeville, PA). Hybridization and washing conditions were as reported (19).

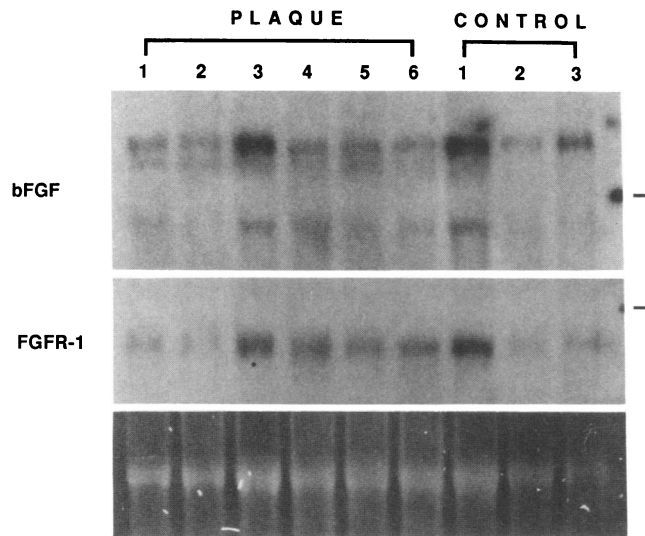
**Reverse transcription (RT).** For RT, 1  $\mu$ g of total RNA from THP-1 cells was incubated in a 10- $\mu$ l reaction mixture as previously described (22). For the other RNA samples from tissues or cultured cells 1  $\mu$ g of total RNA was incubated at 37°C for 1 h in 50  $\mu$ l reverse transcriptase buffer (50 mM Tris-HCl, pH 8.3; 10 mM DTT; 75 mM KCl; 3 mM MgCl<sub>2</sub>; 0.4 mM deoxyribonucleotide triphosphates) containing 20 U of RNasin (Promega Corp., Madison, WI), 0.1  $\mu$ g of random hexamer primers (Boehringer Mannheim Biochemicals, Indianapolis, IN), and 400 U of Moloney murine leukemia virus reverse transcriptase (Gibco/BRL, Gaithersburg, MD). RT was terminated by heating at 95°C for 10 min.

**Qualitative analysis of mRNA levels by RT-PCR.** To answer questions regarding presence of mRNA species, we used PCR amplification at 35 or 40 cycles. A lack of product at these levels of amplification using specific primers generally indicates little or no expression of the gene studied. However, quantitative comparisons between samples are uncertain at these high cycle numbers, because the plateau of expansion has usually been reached (22). Sequence-specific primers used were designed to amplify selectively the messages coding for certain FGF family members, FGF receptors, and human beta-actin (Table I). 1  $\mu$ l of cDNA prepared from THP-1 cells was amplified at 40 cycles as previously described (22) using aFGF, bFGF, and actin primers. 1  $\mu$ l of cDNA prepared from human arteries and vascular cell samples was amplified at 35 cycles in 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.2 mM each deoxyribonucleotide triphosphate, 0.25  $\mu$ g of each primer, and 2.5 U of Taq polymerase (Boehringer Mannheim Biochemicals). Each cycle included denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and primer extension at 72°C for 30 s using a thermocycler (model 9600; Perkin-Elmer Cetus Corp., Norwalk, CT). 10  $\mu$ l of each amplification mixture were electrophoresed on an agarose gel and DNA bands visualized by ethidium bromide staining. In all cases, the estimated sizes of the amplification products detected corresponded well to the predicted sizes. Cloning into the plasmid pCR1000 (Invitrogen Corp., San Diego, CA) and sequencing by the dideoxynucleotide chain termination method using an automated DNA sequencer (model 373A; Applied Biosystems, San Jose, CA) confirmed the identity of FGF-5 and FGF-7 PCR products. Similar analysis of the PCR product obtained using the FGF-3 primer pair indicated no correlation with FGF-3 mRNA. Therefore, data obtained using the FGF-3 primer pair are not reported.

**Immunohistochemistry.** Serial frozen sections (6  $\mu$ m) were cut onto poly-L-lysine coated slides and fixed in acetone. Incubation in 0.3% hydrogen peroxide for 20 min exhausted endogenous peroxidase activity. For each antibody the dilution that yielded optimal specific staining was determined in pilot experiments. Primary antibodies were di-



**Figure 3.** Human atheroma contain aFGF mRNA. This figure shows results of Northern analysis. Five atherosclerotic plaques contained aFGF mRNA. Only one of five control human arteries (carotid artery, lane 1; aortas, lanes 2-4; coronary artery, lane 5) had comparable levels of this message. The major transcript detected in human tissues comigrated with the 4.3-kb species present in RNA from human SMC treated with PMA, used as a positive control. The 3.2- and 2.7-kb transcripts, evident in the positive control, were visualized in extracts of human tissues only at longer exposures. The ethidium bromide-stained gel (bottom) shows that equal amounts of RNA had been used for each sample.



**Figure 4.** Expression of bFGF and FGFR-1 mRNA does not differ between control and atherosclerotic arteries. This figure shows results of Northern analysis. Expression of bFGF mRNA and FGFR-1/*flg* mRNA as detected in six atherosclerotic plaques and three nonatherosclerotic human aortas. Lines at right mark the position of the 28S rRNA; the region of the ethidium bromide-stained gel containing the corresponding 28S band is shown at the bottom.

luted in PBS + 10% horse serum and applied to the sections for 1 h. Following two washes in Tris-buffered saline (TBS) with 2% horse serum, a biotinylated secondary antibody (1:100 dilution; Vector Laboratories, Burlingame, CA) directed against the species of origin of the primary antibody, was applied for 45 min. After two washes with TBS plus 2% horse serum and one in TBS alone, the ABC Elite solution (ABC Elite kit, Vector Laboratories) was applied for 45 min. The substrate 3-amino-9-ethyl-carbazole yielded a brown-red reaction product. Sections were lightly counterstained with methyl green or hematoxylin. The following primary antibodies were used: affinity-purified rabbit polyclonal anti-human aFGF (10 µg/ml; reference 23); mouse monoclonal anti-bovine bFGF (10 µg/ml; Cat. no. 05-117; Upstate Biotechnology Inc., Lake Placid, NY); mouse monoclonal anti-muscle alpha-actin, HHF35 (1:50 dilution; Cat. no. MA-931; Enzo Diagnostics, New York, NY); mouse monoclonal EBM-11 (1:4,000 dilution); mouse monoclonal anti-human von Willebrand factor (1:1,000 dilution, cat. no. M616; Dako Corp.). We also used *Ulex europaeus* agglutinin I (1:800 dilution; Cat. no. L-1060; Vector Laboratories) to identify endothelial cells. Controls were performed using the same concentration of species and isotype-matched antibodies; rabbit polyclonal Ig fraction (Cat. no. X936; Dako Corp.) was used as control for the affinity-purified rabbit polyclonal anti-human aFGF antibody. These irrelevant antibodies did not stain the specimens examined. An additional control for aFGF specificity involved the use of a working dilution of the

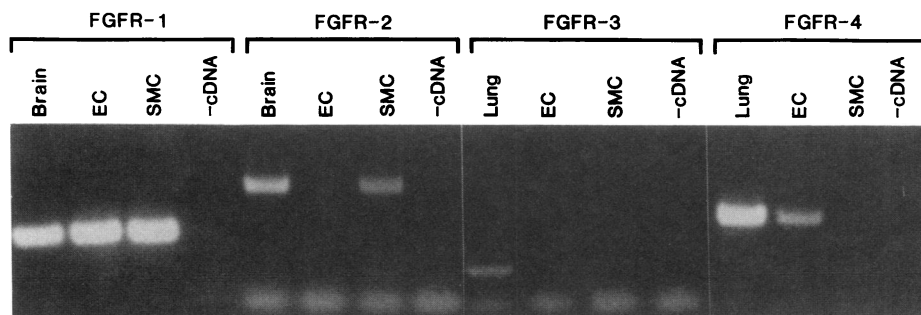
affinity-purified anti-aFGF polyclonal serum after overnight preabsorption with a 100-fold molar excess of human recombinant aFGF. Staining of samples with this serum was completely blocked. Comparative examination of serial sections stained with the above-mentioned antibodies permitted colocalization of growth factors and cell types. We used a semiquantitative grading system to analyze the immunohistochemical results. Two observers independently assigned a histologic score to each specimen, indicating extent of FGF staining associated with a given cell type (EC, SMC, macrophages) on a scale from 0 to 4. Grade 0 indicated that the cell type examined did not stain for the growth factor; grade 1 indicated positivity associated with less than 10% of a given cell type; grade 2 indicated positivity of 10–30% of a given cell type; grade 3 indicated positivity of 30–60% of a given cell type; and grade 4 indicated positivity of more than 60% of a given cell type. Results of this analysis are presented as mean ± SEM. The grades assigned independently by both observers agreed within one grade; differences were resolved by joint examination. Recent hemorrhage was defined as macroscopically visible blood deposit within the vessel wall. Old hemorrhage was defined as presence of hemosiderin, detected using Gomori's Prussian blue reaction identifying ferric ions.

## Results

*Cultured human EC and SMC express aFGF, bFGF, and low levels of FGF-5 mRNA; SMC also express FGF-7 mRNA.* We screened cultured human EC and SMC for expression of FGF family members using RT-PCR at 35 cycles of amplification (Fig. 1). EC and SMC contained aFGF and bFGF mRNA, as previously reported (17), as well as low amounts of FGF-5 mRNA. SMC also expressed FGF-7 mRNA (Fig. 1).

*Control and atherosclerotic human arteries express aFGF, bFGF, and FGF-7 mRNA.* To determine the relevance of these observations in vitro to human atherogenesis, we investigated the expression of FGF family members in control and atherosclerotic human arteries using RT-PCR analysis at 35 cycles (Fig. 2). All control and atherosclerotic human arteries contained aFGF, bFGF, and FGF-7 mRNA. Neither atherosclerotic nor control arteries contained FGF-5 mRNA (Fig. 2), although both EC and SMC expressed this mRNA species in vitro (Fig. 1).

*Human atheroma contain more aFGF mRNA than do control human vessels, but similar levels of bFGF mRNA.* Screening with PCR at 35 cycles of amplification suggested that aFGF and bFGF mRNA were the most abundant FGF transcripts in human arteries. Because quantitation of mRNA levels by PCR has certain limitations (22), we verified the expression of aFGF and bFGF mRNAs in vascular tissue by Northern analysis. This technique detected aFGF mRNA in five of five plaques tested, but only one of five control arteries had comparable levels of this message (Fig. 3). However, Northern analysis showed no consistent difference in the steady state levels of



**Figure 5.** EC in vitro express FGFR-1 and FGFR-4 mRNA. SMC in vitro express FGFR-1 and FGFR-2 mRNA. This figure shows results of RT-PCR analysis at 35 cycles. We detected expression of FGFR-1 and -4 mRNAs in human EC in vitro. Human SMC expressed FGFR-1 and -2 transcripts. Sources of positive control RNA were human brain for FGFR-1 and -2, and human lung for FGFR-3 and -4.

**Table II. Localization of aFGF and bFGF in Human Atheroma and Nonatherosclerotic Arteries**

	EC microvessels	SMC		Macrophages
		Intima	Media	
<b>aFGF</b>				
plaque (n = 14)	3.3±0.7	0.5±0.6	1.1±0.7	2.0±0.6
control (n = 10)	*	0	1.0±0.8	0.9±1.3
<b>bFGF</b>				
plaque (n = 14)	1.5±1.1	1.1±1.0	2.6±0.6	0.5±0.6
control (n = 10)	*	0.5±0.9	3.6±0.7	0

Data are mean±SEM, and represent the histologic scores (percent of cells stained) assigned as follows: Grade 1, < 10%; grade 2, 10–30%; grade 3, 30–60%; and grade 4, > 60%.

\* Control arteries had no intimal microvessels.

bFGF mRNA in control (n = 3) vs. atherosclerotic arteries (n = 6) (Fig. 4).

**Cultured human EC and SMC express FGFR-1 mRNA.** To achieve a more complete understanding of the potential responsiveness of vascular cells to FGFs, we studied the expression of FGFR family members in human cultured vascular EC and SMC using RT-PCR (Fig. 5). SMC contained FGFR-1 mRNA and low levels of FGFR-2 mRNA. EC expressed FGFR-1 transcripts and low levels of FGFR-4 mRNA.

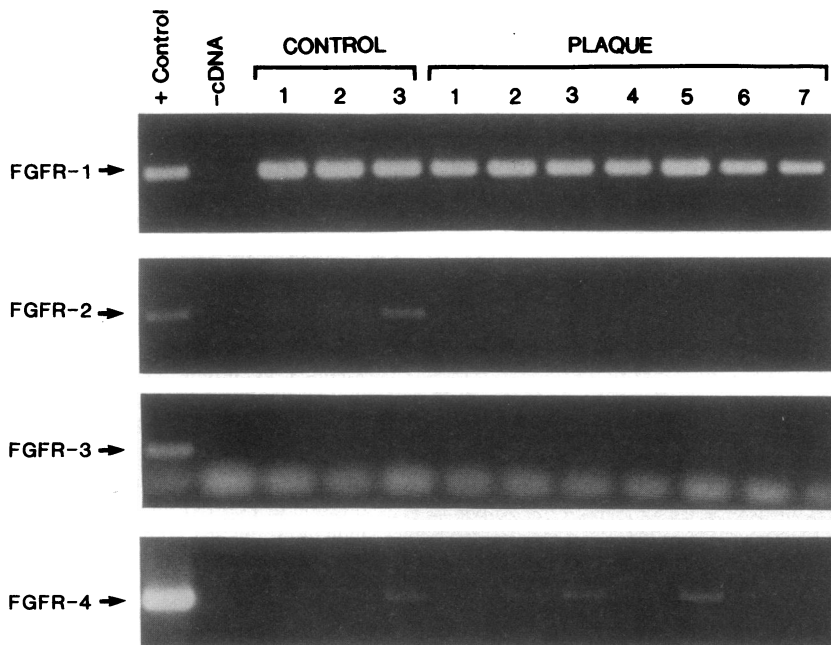
**Control and atherosclerotic human arteries express similar levels of FGFR-1 mRNA.** All control and atherosclerotic samples examined contained similar levels of FGFR-1 mRNA, as detected both by RT-PCR (Fig. 6) and Northern analysis (Fig. 4). In addition, only control arteries contained FGFR-2 mRNA, while some control or atherosclerotic specimens contained FGFR-4 mRNA (Fig. 6).

**Neovascularization and hemorrhages within plaques.** The intima of all plaques (n = 14) contained microvascular plexi.

These vessels generally had a thin wall composed of EC alone or with one or two layers of SMC (Fig. 7). Microvascular plexi were found mainly at the base of plaque near the junction between intima and media (15/15 samples), or near the necrotic core (14/15 samples). These vessels localized within or adjacent to areas of macrophage infiltration in 13/15 samples. Nine plaques showed recent hemorrhages (9/15). All three plaques examined with Gomori's test had signs of old hemorrhages. In all three cases, the hemosiderin deposits were located near microvessels within lesions (data not shown). The specimens studied showed no areas of ulceration or plaque rupture.

**Distribution of aFGF and bFGF in atherosclerotic and control arteries.** Because aFGF and bFGF mRNA were the most abundant FGF transcripts in human arteries, we immunolocalized the corresponding proteins using specific antibodies. The EC lining the luminal surface of plaques or control arteries were sometimes incompletely preserved due to perioperative manipulation, but whenever present stained for aFGF both in control and atherosclerotic arteries (Fig. 7 and 8). EC of lesional microvessels consistently stained for aFGF (Fig. 7, Table II). Medial SMC stained weakly for aFGF in both control and atherosclerotic arteries (Figs. 7 and 8; Table II). Intimal SMC in plaques had even less aFGF staining than medial SMC of both control arteries and atheroma (Figs. 7 and 8; Table II). Macrophage-rich areas within plaques stained for aFGF in 13/15 atherosclerotic arteries (Fig. 7, Table II). Also, scattered macrophages present in the thickened intima of control arteries colocalized with aFGF protein, but with less intense staining (Table II).

Luminal EC, where preserved, stained for bFGF in both control and atherosclerotic arteries (Figs. 8 and 9). EC of plaque microvessels inconsistently stained for bFGF protein in 12/15 samples (Table II). Medial SMC colocalized with bFGF in all control and atherosclerotic arteries (Figs. 8 and 9; Table II). Basic FGF staining also coincided with microvascular SMC of vasa vasorum (Fig. 9, Table II). However, intimal SMC, identified by staining for muscle alpha-actin, often



**Figure 6.** Expression of mRNA for FGFR family members in human carotid plaques and control arteries. This figure shows results of RT-PCR analysis at 35 cycles. All control and atherosclerotic arteries examined contained FGFR-1 transcripts. Only control arteries had FGFR-2 mRNA, while FGFR-4 mRNA expression was inconsistent. Sources of positive control RNA were as indicated for Fig. 5.

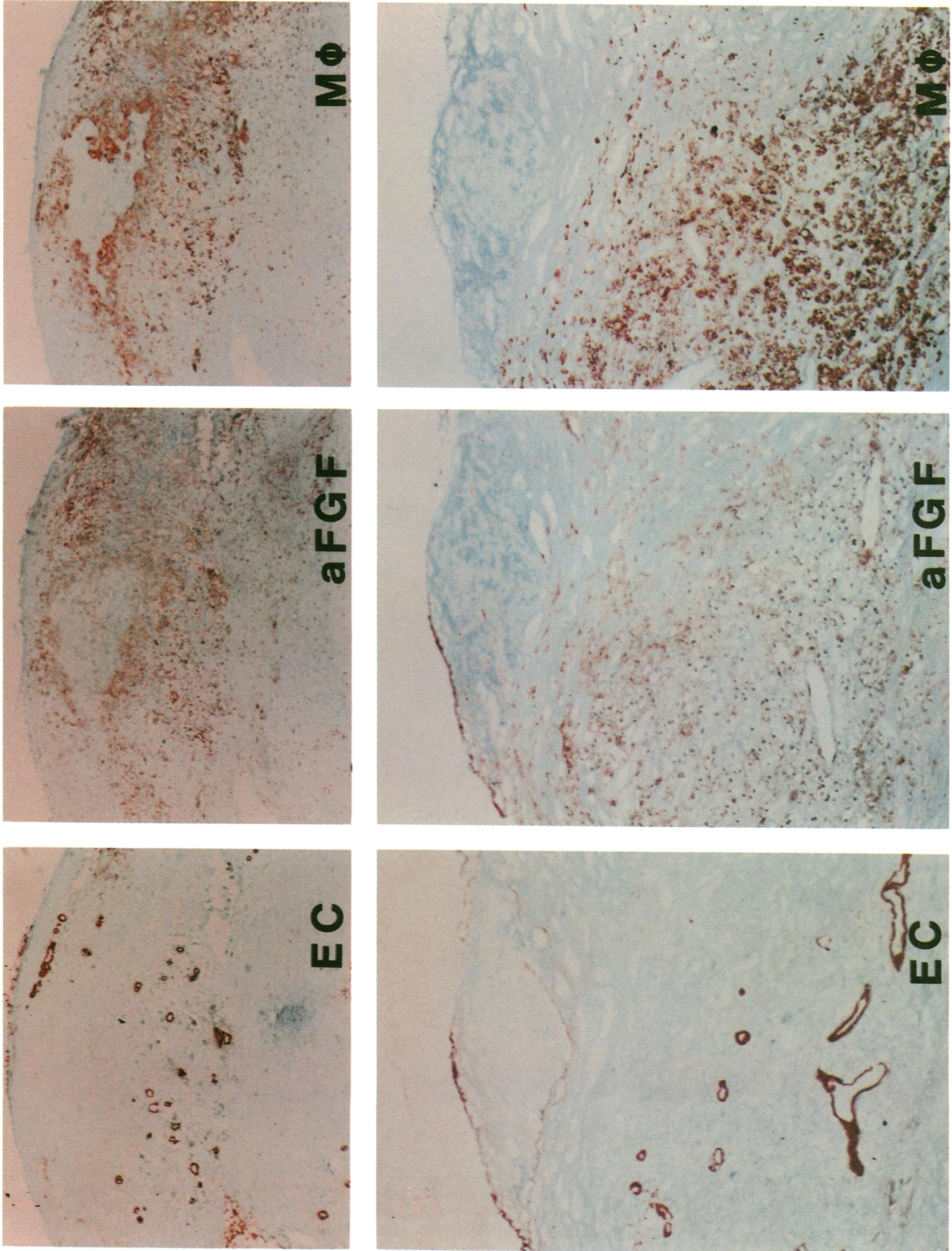
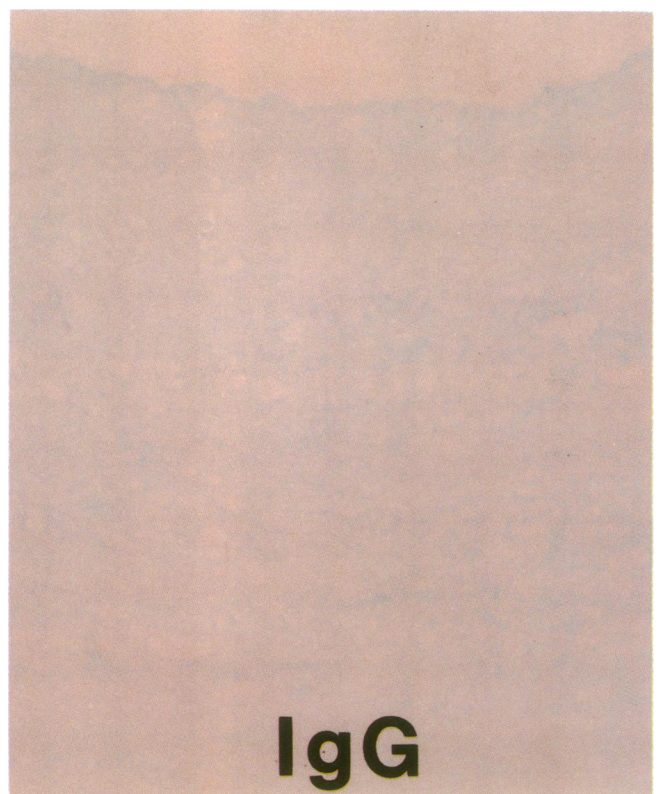
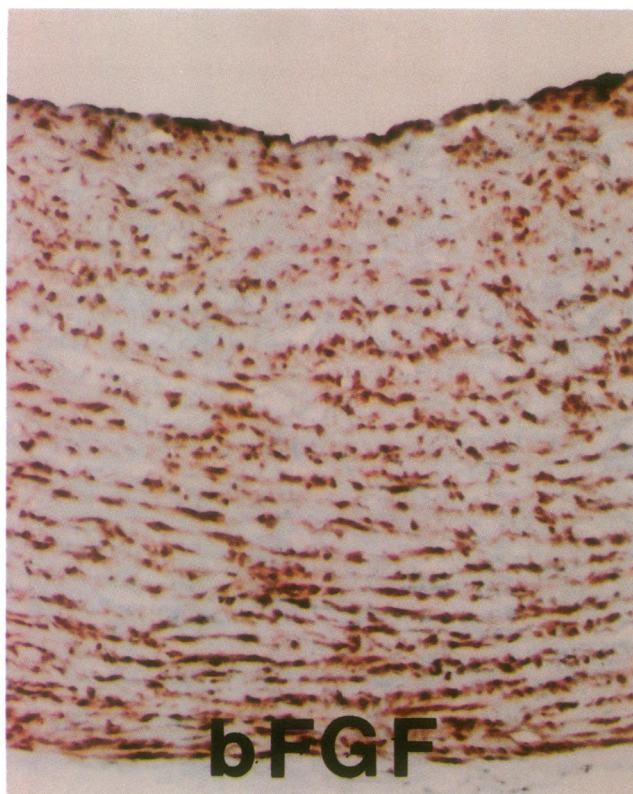
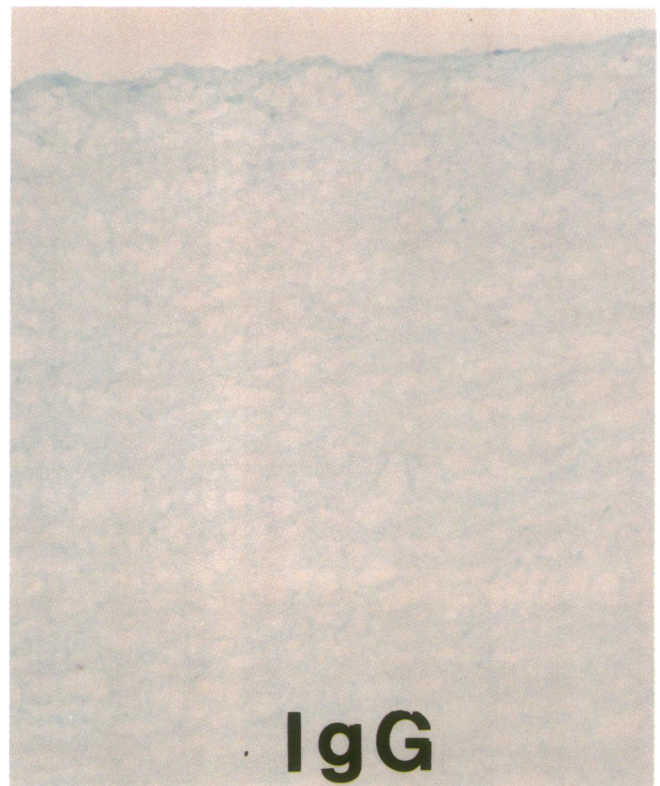
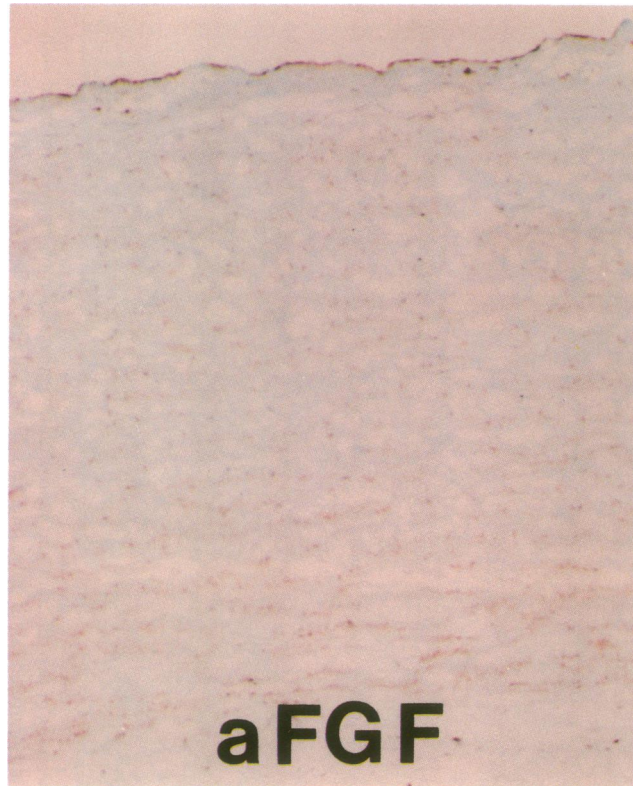
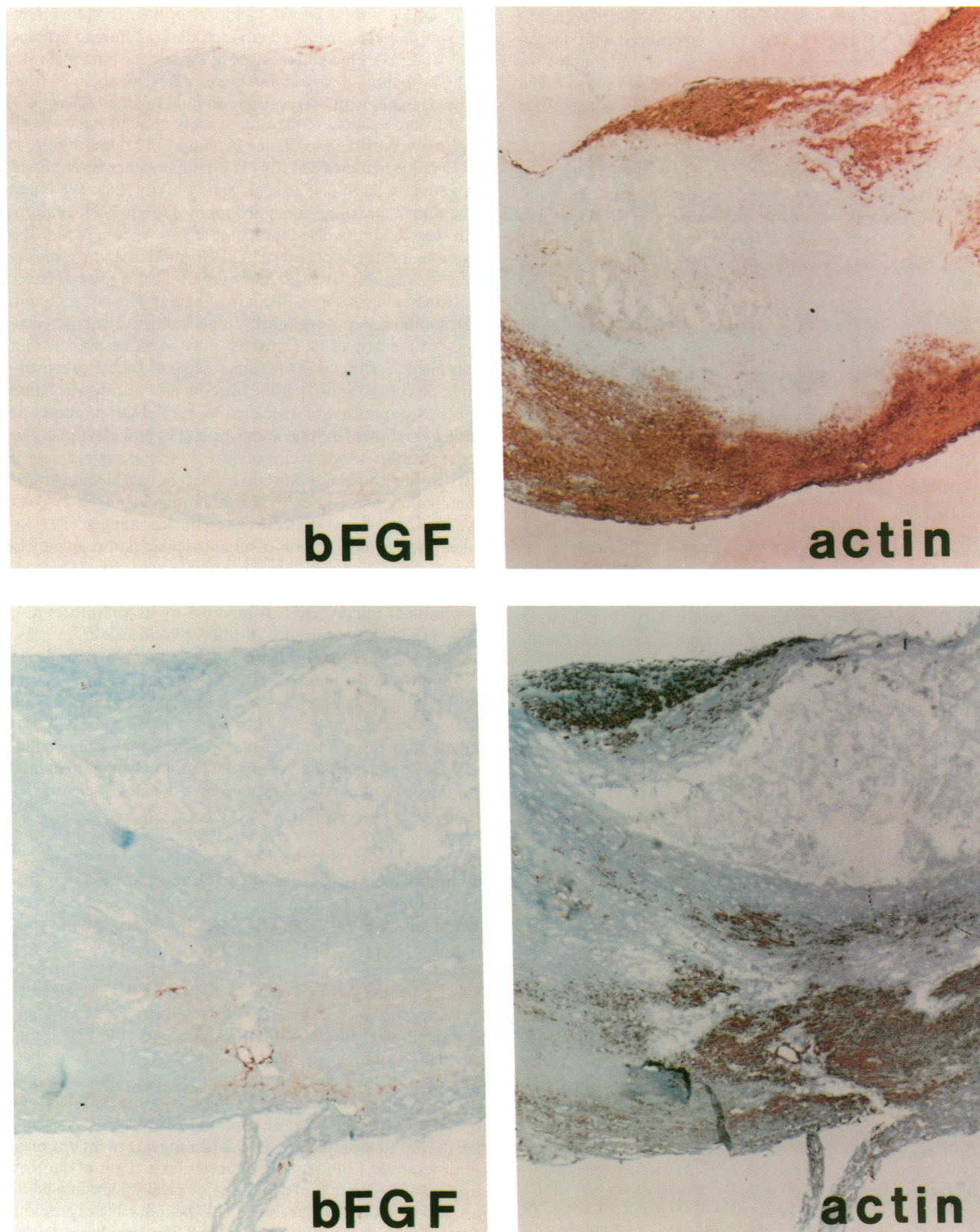


Figure 7. Atherosclerotic plaques contain abundant microvessels and high levels of aFGF protein. Serial sections of two different and representative atherosclerotic plaques, oriented with adventitial side (*upper row*;  $\times 40$ ) or luminal side (*lower row*;  $\times 100$ ) on top. Immunodetectable aFGF (affinity-purified rabbit polyclonal anti-human aFGF antibody (10  $\mu\text{g}/\text{ml}$ ; reference 23) colocalized with luminal and microvascular EC, identified with *Ulex europaeus* agglutinin I (1:800 dilution), as well as with areas of macrophage infiltration (M $\Phi$ ), stained with the EBM-11 antibody (1:4,000 dilution). Note the regional distribution of aFGF.



**Figure 8.** Control human arteries contain scarce aFGF protein but high levels of bFGF protein. Serial sections of nonatherosclerotic human arteries oriented to show the lumen in the upper portion of each picture. Immunodetectable aFGF, identified by staining with the affinity-purified rabbit polyclonal anti-human aFGF (10  $\mu\text{g}/\text{ml}$ ; reference 23) colocalized with luminal EC, recognized by staining with *Ulex europaeus* agglutinin I (not shown), and with scattered medial SMC of human aorta, characterized by staining with HHF35 antibody (not shown). Substantial bFGF protein, identified with a mouse monoclonal anti-bovine bFGF antibody (10  $\mu\text{g}/\text{ml}$ ), colocalized with EC and with medial SMC of human carotid (lower row;  $\times 100$ ).





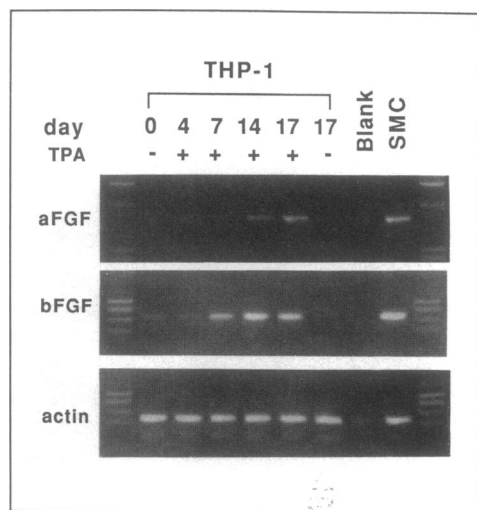
**Figure 9.** Atherosclerotic plaques contain low levels of bFGF protein. Serial sections of two different and representative plaques, oriented with lumen on top and shown at  $\times 40$  (upper row) or  $\times 100$  (lower row). Immunodetectable bFGF, identified with a mouse monoclonal anti-bovine bFGF antibody ( $10 \mu\text{g/ml}$ ), colocalized with luminal EC, recognized by staining with *Ulex europaeus* agglutinin I (1:800 dilution). Areas with intimal SMC, localized by muscle alpha-actin staining (not shown) contain little immunodetectable bFGF protein.

stained less intensely for bFGF (Table II), and three plaques lacked any immunoreactive bFGF protein associated with intimal SMC (3/15; Fig. 9). Basic FGF colocalized with macrophages within atherosclerotic plaques (6/15), but staining for this protein was limited to less than 10% of cells (Table II). The few intimal macrophages in control arteries did not stain for bFGF (Table II).

*THP-1 cells inducibly express aFGF mRNA.* Because aFGF protein consistently colocalized with plaque macrophages, a cell type not heretofore known to express this gene, we tested whether mononuclear phagocytes could express the aFGF gene in vitro. Monocytes, monocyte-derived macrophages, and THP-1 cells, treated in vitro with PMA, IFN-gamma, LPS, lysophosphatidylcholine, or PDGF-BB for up to 72 h contained no aFGF mRNA (data not shown). However, prolonged treatment (14–17 d) of THP-1 cells with PMA induced accumulation of aFGF mRNA (Fig. 10). Unstimulated THP-1 cells at days 0 or 17 of culture did not contain this message. PMA-treated THP-1 cells also expressed bFGF mRNA at days 7, 14, and 17 of stimulation, while unstimulated THP-1 cells at days 0 and 17 lacked this message (Fig. 10). Two independent experiments yielded similar results.

## Discussion

Experimental studies in rats (13, 14) indicate a role for bFGF in initiating medial SMC proliferation after balloon injury. aFGF may also accelerate re-endothelialization and reduce intimal thickening after a similar type of injury (12). Despite considerable interest in the role of FGFs in vascular growth,



**Figure 10.** Human monocytoic THP-1 cells express aFGF and bFGF genes inducibly in vitro. This figure shows results of RT-PCR at 40 cycles of amplification. THP-1 cells were continuously stimulated with PMA (100 nM) for up to 17 d to mimic macrophage differentiation. Selective primers for aFGF, bFGF, and beta-actin were used. Treatment with PMA induced expression of aFGF mRNA at days 14 and 17, and of bFGF mRNA at days 7, 14, and 17 of stimulation. Unstimulated cells at days 0 and 17 contained neither aFGF nor bFGF transcripts. Human SMC treated with PMA served as a positive control; equal volumes of the reaction mixture without addition of cDNA provided negative controls (Blank). PCR products obtained using beta-actin primers show that similar amounts of cDNA were used and similarly amplified.

there is little information available regarding the presence and distribution of these growth factors or of their receptors in either normal (15, 16) or atherosclerotic (16) human arteries. This study examined these issues to explore the potential roles of FGFs in human vessels, and to integrate data obtained from animal studies with observations on human atherosclerosis.

Advanced human atheroma usually contain microvessels that may contribute to plaque evolution or complication via intraplaque hemorrhages (24–27). Human atherosclerotic tissues contain thermolabile angiogenic activity (28). We found that human atheroma contained more aFGF mRNA and protein than did uninvolved human arteries. We also observed a prominent association between aFGF protein and plaque regions rich in microvessels, suggesting that this growth factor participates in neovascularization of lesions. However, protein distribution does not necessarily imply biological activity, especially in the case of FGFs, which may be sequestered within cells or by binding to extracellular heparan sulfate proteoglycans. The observation that the introduction of constructs that direct the expression of a secreted form of aFGF promotes de novo formation of intimal microvessels (7) provides direct evidence that this growth factor can cause intimal angiogenesis in vivo. Furthermore, Gordon and colleagues have demonstrated that dividing cells within human atheroma, identified by positive staining for the proliferating cell nuclear antigen (29), localize preferentially in areas of microvascularization, an indication of ongoing growth factor activity in these loci (Gordon, D., unpublished observations). Hemosiderin deposits located near intimal microvessels, as reported by us and others (24, 25), suggest the occurrence of prior hemorrhage from these structures. The newly formed vascular channels may be prone to rupture; consequent thrombosis in situ might activate thrombin-mediated events, such as SMC proliferation. Plaque microvessels also provide a large surface area, which can promote further recruitment of leukocytes via expression of adhesion molecules (30) and/or production of chemoattractants, and thus contribute to the evolution of the atherosclerotic lesion.

We found that regions in plaques with abundant aFGF and microvessels often contained numerous macrophages. The colocalization of macrophages and immunoreactive aFGF in human atheroma was intriguing, since mononuclear phagocytes are not known to produce aFGF. We investigated this possibility using THP-1 cells, a homogeneous and well characterized monocytoic cell line adapted to long-term culture. Interestingly, induction of aFGF mRNA did not occur upon short-term exposure to a wide range of stimuli, but required long-term treatment with PMA. Phorbol esters promote THP-1 differentiation into macrophage-like cells (19) and induce expression of FGF genes in other human cell types (17, 18). The requirement for prolonged stimulation may mimic in vitro the shift in phenotype of mononuclear phagocytes occurring during atherogenesis in vivo. Macrophages in lesions may be far removed from blood monocytes, as a result of chronic stimulation by a variety of pathophysiologically relevant mediators, modeled here by phorbol ester. The inducible expression of the aFGF gene by a monocytoic cell line, together with the observation that in vivo plaque macrophages localize near or within areas rich in microvessels, suggests that these cells may contribute to the neovascularization characteristic of advanced atherosclerotic lesions by local release of angiogenic substances such as aFGF.

Macrophages can also produce other angiogenic factors (31–33), such as bFGF (33), in response to various stimuli (31). Our analysis of human atherosclerotic samples showed less consistent colocalization of bFGF than of aFGF with plaque macrophages. However, we found substantial bFGF in the endothelium and in the medial layer of nonatherosclerotic human arteries, as previously reported in normal rat arteries (13). Balloon withdrawal injury to the normal rat carotid causes intimal thickening resembling in some respects the restenotic lesion of human arteries after angioplasty. Experimental data implicate bFGF in the initiation of medial SMC proliferation during this process in rats (11, 13). However, because human atheroma contain scarce bFGF protein, as indicated here and elsewhere (16), balloon injury to an advanced human lesion may release less bFGF than would injury to normal rat arteries. Our finding of scant bFGF protein in human atherosclerotic intima parallels the low levels of bFGF found in intimal lesions of balloon-injured rat carotid arteries (13).

Although human atherosclerotic arteries contained less immunoreactive bFGF protein than control vessels, levels of bFGF mRNA did not differ significantly. Several factors could account for this apparent disparity between bFGF mRNA and protein levels. For example, the regulation of bFGF synthesis in atherosclerotic lesions might be posttranscriptional. Alternatively, ongoing biosynthesis of bFGF, reflected in mRNA levels, may not contribute substantially to the steady state level of bFGF protein, as the bulk of this growth factor present in normal arteries may represent preformed intracellular or matrix-bound protein. The low level of bFGF protein documented in plaques could derive from depletion or consumption of bFGF stores rather than from reduced biosynthesis.

With respect to more recently recognized forms of FGFs, we found consistent expression of FGF-7 mRNA both in cultured SMC and in human arteries. FGF-7, also known as keratinocyte growth factor (34), selectively stimulates proliferation of epithelial cells (35) but has no mitogenic activity on human EC (35) or SMC (Rubin, J., personal communication). Furthermore, we found that human vascular cells and arteries do not contain mRNA for the FGFR-2 splice variant that specifically binds FGF-7 (Winkles, J., unpublished observations). Therefore, the biological relevance of FGF-7 within human arteries remains uncertain.

Growth factor responsiveness requires expression of an appropriate receptor. FGFRs constitute a family encoded by at least four different genes (36–39). Alternative splicing permits the assembly of multiple isoforms of each receptor (9, 10). Furthermore, each FGFR can bind two or more FGFs, with graded affinity. FGFR-1, encoded by the *flg* gene, can bind both aFGF and bFGF. We found consistent expression of FGFR-1 mRNA in all control and atherosclerotic arteries examined. Several investigators have explored the use of toxins conjugated to FGFs as a strategy to prevent excessive proliferation of SMC after arterial injury. Our data suggest that this approach might not spare EC and SMC in normal human arteries, a point that merits further investigation.

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