Proliferation of Neointimal Smooth Muscle Cells after Arterial Injury

DEPENDENCE ON INTERACTIONS BETWEEN FIBROBLAST GROWTH FACTOR RECEPTOR-2 AND FIBROBLAST GROWTH FACTOR-9*

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The growth factor signaling mechanisms responsible for neointimal smooth muscle cell (SMC) proliferation and accumulation, a characteristic feature of many vascular pathologies that can lead to restenosis after angioplasty, remain to be identified. Here, we examined the contribution of fibroblast growth factor receptors (FGFRs) 2 and 3 as well as novel fibroblast growth factors (FGFs) to such proliferation. Balloon catheter injury to the rat carotid artery stimulated the expression of two distinctly spliced FGFR-2 isoforms, differing only by the presence or absence of the acidic box, and two distinctly spliced FGFR-3 isoforms containing the acidic box and differing only by the presence of either the IIIb or IIIc exon. Post-injury arterial administration of re-combinant adenoviruses expressing dominant negative mutant forms of these FGFRs were used to assess the roles of the endogenous FGFR isoforms in neointimal SMC proliferation. Dominant negative FGFR-2 containing the acidic box inhibited such proliferation by 40%, whereas the dominant negative FGFR-3 forms had little effect. Expression of FGF-9, known to be capable of binding to all four neointimal FGFR-2/-3 isoforms, was abundant within the neointima. FGF-9 markedly stimulated both the proliferation of neointimal SMCs and the activation of extracellular signal-related kinases 1/2, effects which were abrogated by the administration of antisense FGF-9 oligonucleotides to injured arteries and the expression of the dominant negative FGFR-2 adenovirus in cultured neointimal SMCs. These studies demonstrate that, although multiple FGFRs are induced in neointimal SMCs following arterial injury, specific interactions between distinctly spliced FGFR-2 isoforms and FGF-9 contribute to the proliferation of these SMCs.

Atherosclerosis, vasculopathies, post-transplant arteriosclerosis, and restenosis are characterized by expansion of the arterial intima as a result of the infiltration of mononuclear leukocytes, the accumulation of the extracellular matrix, and the proliferation of vascular smooth muscle cells (SMCs) (1–3).

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1 The abbreviations used are: SMC, smooth muscle cell; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; BrdUrd, bromodeoxyuridine; RT, reverse transcription; DN, dominant negative; GFP, green fluorescent protein; ERK, extracellular signal-related kinase.
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-2, and -3 involves the alternate usage of either the "IIb" or "IIIC" exon to encode the carboxyl-terminal portion of Ig-like domain III (14). Expression of these FGFR isoforms appears to be regulated in a tissue-specific manner, with IIb exon-containing isoforms apparently restricted in expression to epithelial cell lineages and IIc exon-containing isoforms restricted in expression to mesenchymal cell lineages (17). As a consequence of such splicing events, FGF ligands can have either a diverse or an extremely restricted FGFR binding profile. For example, FGF-1 binds to all FGFR isoforms with high affinity, FGFR-2 exhibits a high affinity for binding to FGFR-1IIb, FGFR-1IIIc, and FGFR-3IIc, and FGFR-9 exhibits a high affinity for binding to FGFR-2IIb, FGFR-3IIb, and FGFR-3IIc (17); conversely, FGF-7 and FGF-10 are restricted in binding, with high affinity only to FGFR-2IIb (14, 17, 18). Finally, it has become increasingly apparent that the cellular potency of FGFR signaling is also influenced by post-translational receptor modification, in particular the enhanced mitogenic capacity of FGFR-2β isoforms arising from glycosaminoglycan modification at the acidic box motif (19).

Here, we demonstrate that arterial injury induces the predominant expression by neointimal SMCs of two distinctly spliced FGFR-2IIIC β-isomers, differing from each other only by the presence/absence of the acidic box motif, and two distinctly spliced FGFR-3 α-isomers, both containing the acidic box motif and differing from each other by the alternate IIIb/IIIc composition of Ig-like domain III. Using adenoviruses expressing dominant negative mutants of specific receptor types truncated at their transmembrane domain and devoid of tyrosine kinase activity, we demonstrate that signaling through FGFR-2IIIC is a major contributor to neointimal SMC proliferation. We further demonstrate that a specific FGF ligand, FGF-9, known to have high affinity for the neointimal FGFR-2-3 isoforms, shows an elevated neointimal expression after arterial injury and contributes to neointimal SMC proliferation through an interaction with FGFR-2IIIC. These findings, for the first time, demonstrate a major regulatory role for an FGFR2-IIIc/FGF-9 interaction in the stimulation of neointimal SMC proliferation in the injured arterial wall.

EXPERIMENTAL PROCEDURES

Carotid Artery Injury—Balloon catheter injury to the rat carotid artery was carried out as described previously (4, 20). Briefly, after anesthetizing the rats with a mixture of pentobarbionate (30 mg/kg), methohexitone (40 mg/kg), and atropine sulfate (3 mg/kg) administered by intraperitoneal injection, a 2F Fogarty arterial embolectomy catheter (Baxter) was passed through an arteriotomy in the left common carotid artery to its bifurcation. The femoral artery was then ligated, the incision made, and the animal was allowed to recover for 2–14 days after the first surgical procedure (see below) or were sacrificed 2 to 14 days later with an overdose of pentobarbitone (100 mg/kg intraperitoneally). At 1 week, a second surgical procedure (see below) or were sacrificed 2 to 14 days later with an overdose of pentobarbitone (100 mg/kg intraperitoneally). A further two times. The femoral artery was then ligated, the incision made, and the animal was allowed to recover in a humidified, warming chamber for 1–2 h. The ligatures and catheter were then removed, the external carotid ligated, the incision closed, and the animal allowed to recover (see above). To measure cell proliferation in balloon-injured vessels, 50 μg/kg tritiated thymidine (BrdUrd) was administered by intraperitoneal injection 24 and 8 h before sacrifice.

Tissue Collection and Immunohistochemistry—Carotid arteries were removed from the sacrificed animals and cleaned of non-vascular tissue. Then, arteries intended for mRNA analyses or Western blotting underwent careful removal of their neointima under a dissecting microscope, and the neointimal tissue was then rapidly frozen in liquid nitrogen. Carotid arteries for immunohistochemical analyses were mounted in OCT compound (Tissue Tek; Miles Scientific), frozen in pentane over liquid nitrogen, and stored at −70 °C. Immunohistochemical detection of FGFR and FGF peptide expression and BrdUrd incorporation was carried out using 6 μm of frozen cross-sections and the avidin-biotin- peroxidase complex kit (Vector Laboratories) with 3,3′-diaminobenzidine tetrachloride as the chromogenic substrate, essentially as described previously (21). Primary antibodies to FGFR-2 (1:500, rabbit anti-human polyclonal; catalog number sc-122), FGFR-3 (1:500, rabbit anti-human polyclonal; catalog number sc-123), and FGF-9 (1:500, goat anti-human polyclonal; catalog number sc-1368) were from Santa Cruz Biotechnology Inc., the anti-FGFR-1 antibody (1:500, rabbit anti-bovine polyclonal; catalog number G5081) was from Promega, the anti-FGF-2 antibody (1:500, mouse anti-human monoclonal; catalog number DE6) was from du Pont de Nemours and Company, and the anti-BrdUrd antibody (1:100, mouse monoclonal; catalog number 03-3900) was from Zymed Laboratories Inc.. The appropriate peroxidase-labeled secondary antibodies (Vector Laboratories) were used and the slides were exposed for 1:200. After completing the immunohistochemical procedures, the sections were lightly counterstained with hematoxylin.

Reverse Transcription PCR—RNA was isolated from 2–3 neointima samples and treated with DNase (Promega) essentially as described previously (21). Approximately 300 ng of RNA was reverse transcribed using a GeneAmp RNA-PCR kit (PerkinElmer Life Sciences), and specific FGFR-2, FGFR-3, and FGF-9 fragments were amplified using 35 PCR cycles (see next paragraph) as described previously (20). For determination of FGFR cDNA structures and verification of FGF-9 amplification specificity, nucleotide sequencing (TaqTrack, Promega) was carried out on gel-purified fragments cloned into pGEM-T vectors (Promega).

Oligonucleotide Primers for RT-PCR and Antisense FGF-9 Oligonucleotides—FGFR-2 and FGFR-3 primers were designed to provide definitive structural information on distinct regions of the receptors encoded by each amplified cDNA (see Fig. 2A). Specifically, to detect the presence/absence of Ig-like domain I and the acidic box motif in the extracellular region of each receptor, primer sets designated as "Region A" were utilized in which the sense and antisense primers were chosen such that they were sufficiently distal from the 5′ and 3′ splicing boundaries of both consecutive exons that code for Ig-like domain I and the acidic box; for detection of alternatively spliced IIb or IIc exons in the carboxyl terminus of Ig-like domain III and in the presence of the transmembrane-spanning domain, primer sets designated as "Region B-TM" in the case of FGFR-2 and "Region A-IIb", "Region A-IIc", or Region B-TM in the case of FGFR-3 were utilized. The precise location of each of the primer pairs within each cDNA and the predicted size of their amplification products were as follows. For FGFR-2 cDNA (GenBank™ accession numbers Z61536, L19104, and D83498), in Region A the sense primer comprised nucleotides 19–42 and the antisense primer comprised nucleotides 558–614 (596 bp); in Region B-TM the sense primer comprised nucleotides 884–913 and the antisense primer comprised nucleotides 1426–1445 (562 bp). To detect the presence in this cDNA fragment of the IIb or IIc exon, the restriction endonuclease HpaI, a site for which is present in the IIc exon but not the IIb exon, was used to digest the amplified product. For FGF-3 cDNA (GenBank™ accession numbers M81342 and L26492), in Region A the sense primer comprised nucleotides 1696–1722 (26 bp) and specific antisense primer (Region A-IIc, nucleotides 956–981; 976 bp). For the transmembrane-spanning domain (Region B-TM), the sense primer comprised nucleotides 913–942 and the antisense primer comprised nucleotides 1676–1698 (842 bp). To detect FGF-9 cDNA (GenBank™ accession number D14839), the sense primer comprised nucleotides 1696–1722 (26 bp) and specific antisense primer (Region A-IIc, nucleotides 956–981; 976 bp). For the transmembrane-spanning domain (Region B-TM), the sense primer comprised nucleotides 913–942 and the antisense primer comprised nucleotides 1676–1698 (842 bp). To detect FGF-9 cDNA (GenBank™ accession number D14839), the sense primer comprised nucleotides 1696–1722 (26 bp) and specific antisense primer (Region A-IIc, nucleotides 956–981; 976 bp). For the transmembrane-spanning domain (Region B-TM), the sense primer comprised nucleotides 913–942 and the antisense primer comprised nucleotides 1676–1698 (842 bp). To detect FGF-9 cDNA (GenBank™ accession number D14839), the sense primer comprised nucleotides 1696–1722 (26 bp) and specific antisense primer (Region A-IIc, nucleotides 956–981; 976 bp). For the transmembrane-spanning domain (Region B-TM), the sense primer comprised nucleotides 913–942 and the antisense primer comprised nucleotides 1676–1698 (842 bp). To detect FGF-9 cDNA (GenBank™ accession number D14839), the sense primer comprised nucleotides 1696–1722 (26 bp) and specific antisense primer (Region A-IIc, nucleotides 956–981; 976 bp). For the transmembrane-spanning domain (Region B-TM), the sense primer comprised nucleotides 913–942 and the antisense primer comprised nucleotides 1676–1698 (842 bp). To detect FGF-9 cDNA (GenBank™ accession number D14839), the sense primer comprised nucleotides 1696–1722 (26 bp) and specific antisense primer (Region A-IIc, nucleotides 956–981; 976 bp). For the transmembrane-spanning domain (Region B-TM), the sense primer comprised nucleotides 913–942 and the antisense primer comprised nucleotides 1676–1698 (842 bp).

In Situ RT-PCR—To detect FGF-9 mRNA in the neointima, 6-μm
cross-sections of carotid arteries fixed onto gelatinized slides with formalin were subjected to in situ RT-PCR as described previously (23). Briefly, FGF-9 mRNA was detected by a "one-step" RT-PCR method (35 cycles) utilizing EZ taq polymerase (PerkinElmer Life Sciences) according to the manufacturer's protocol and digoxigenin-11-dUTP. Messenger RNA expression/localization was then determined with an anti-digoxigenin antibody coupled to alkaline phosphatase; color detection was carried out using the substituted nitro blue tetrazolium and blue tetrazolium salt. Paraffin-embedded tissue sections not exposed to PCR primers and reaction mixtures were used.

Recombinant Dominant Negative FGFR-2 and FGFR-3 Adenoviruses—The pAdEasy-1 virus and shuttle plasmid pAd-TrackCMV were obtained from Dr. R. Hannan, Peter MacCallum Cancer Institute, Melbourne, Australia (24). Adenoviruses expressing dominant-negative (DN) FGFR-2 IIIbAB (devoid of Ig-like domain I) and containing the acidic box), FGFR-3 IIIAB, and FGFR-3 IIIcAB (both containing all three Ig-like domains and the acidic box and differing from each other only in the alternate presence of either the IIIb or IIIc exon in the carboxyl terminus of Ig-like domain III) were prepared by amplifying their cDNAs from rat SMC RNA and truncating these cDNAs at the transmembrane domain with the inclusion of a FLAG epitope tag immediately distal to the last amino acid of the transmembrane domain. These cDNAs were cloned into pAd-TrackCMV using the following PCR primers incorporating KpnI and XbaI restriction sites: FGFR-2 IIIbAB, 5'-AGCAGCTGATGTTACGTGGGGGCTCTTCATG-3' (sense; nucleotides 1–24 of cDNA; KpnI site is lowercase and underlined and the initiation codon is bold and italicized) and 5'-CCGGTTGAGTCCCTCCTTAGTCCG-3' ('antisense; nucleotides 1207–1236 of cDNA; XbaI site is lowercase and underlined, termination codon is bold and italicized, and the FLAG epitope is in small caps); and FGFR-3 IIIbAB or FGFR-3 IIIcAB, 5'-GCAAGCTTATGTTACGTGGGGGCTCTTCATG-3' (sense; nucleotides 1–24 of cDNA; KpnI site is lowercase and underlined and the initiation codon is bold and italicized) and 5'-CCGGTTGAGTCCCTCCTTAGTCCG-3' ('antisense; nucleotides 1180–1209 of cDNA; XbaI site is lowercase and underlined, the termination codon is bold and italicized, and the FLAG epitope is in small caps). The integrity of each construct was confirmed by automated DNA sequencing, and the differentiation between either the FGFR-3 IIIbAB or the FGFR-3 IIIcAB constructs was confirmed by the use of restriction enzymes specific for either the IIIb or IIIc exon. Recombinant adenoviruses were generated by bacterial homologous recombination between pAd-TrackCMV containing DN-FGFR cDNAs and pAdEasy-1. Large scale amplification and purification using human embryonic kidney 293 cells was then carried out as described previously (25).

Neointimal SMC Culture and Proliferation Assay—Neointimal SMCs were isolated by elastase/collagenase digestion of 2–3 neointimas from healing carotid arteries and cultured as described previously (26). These SMCs exhibited the "cobblestone" morphology described previously for such SMCs (26). Proliferation stimulated by FGF-9 was determined on semiconfluent neointimal SMC cultures that possess unique FGF binding properties (14, 17), we initially investigated the expression of two FGFRs, FGFR-2 and FGFR-3, in the rapidly proliferating SMCs within the growing neointima of healing rat carotid arteries injured with an inflated balloon catheter. Ten days after injury, high concentrations of FGFR-2 and FGFR-3 were expressed in the neointima and the media of these arteries (Fig. 1, A and B). Four days later, FGFR-2 levels were greatly reduced (Fig. 1C), but not in uninjured carotid arteries (Fig. 1E and F). To gain an insight into the potential role of these receptors in neointimal SMC proliferation and the FGF ligands with which they might interact, we determined the expression of distinctively spliced FGFR-2 and FGFR-3 isoforms by RT-PCR analysis of their mRNAs isolated from the neointima, followed by nucleotide sequencing of specific amplified fragments. Analysis of FGF-2 mRNA was performed using oligonucleotide primers (depicted in Fig. 2A) encompassing the extracellular region that includes Ig-like domains I and II (Region A, nucleotides 19–614 of the rat cDNA) and the region that includes Ig-like domain III and the transmembrane-spanning domain (Region B-TM, nucleotides 884–1445 of the rat cDNA). Amplification using Region A primers indicated that only trace amounts of full-length FGF-2 containing all three Ig-like domains was expressed, as shown by the low abundance of the expected PCR product (596 bp; Fig.

nases (ERK 1/2) in the neointimal SMCs, either with or without prior DN-FGFR-2 infection, was assessed with Western blot analysis using a mouse anti-human monoclonal phospho-p42/44 mitogen-activated protein kinase (Thr202/Tyr204) antibody (Cell Signaling Technology catalog number 9106); in these SMCs total ERK levels were assessed using a rabbit anti-human polyclonal antibody to ERK 1/2 (Santa Cruz Biotechnology Inc. catalog number sc-93), and levels of DN-FGFR-2 IIIb-AB and IIIc-AB were assessed using the anti-FLAG antibody.

Statistical Analysis—Results are expressed as mean ± S.E. The inhibitory effect of the DN-FGFR adenoviruses and the antisense-FGF-9 oligonucleotide on neointimal SMC proliferation in injured arteries was assessed using unpaired t tests. Significance was established by a value of p < 0.05.

RESULTS

FGFR Expression in Proliferating SMCs of the Neointima—Because FGFRs bind FGF family members with varying affinities, and alternative hnRNA splicing leads to receptor isoforms that possess unique FGF binding properties (14, 17), we initially investigated the expression of two FGFRs, FGFR-2 and FGFR-3, in the rapidly proliferating SMCs within the growing neointima of healing rat carotid arteries injured with an inflated balloon catheter. Ten days after injury, high concentrations of FGFR-2 and FGFR-3 were expressed in the neointima and the media of these arteries (Fig. 1, A and B). Four days later, FGFR-2 levels were greatly reduced (Fig. 1C), but not FGFR-3 continued to be expressed (Fig. 1D).

FIG. 1. FGFR-2 and FGFR-3 expression in the injured rat carotid artery. Immunohistochemical detection of FGFR-2 and FGFR-3 in a carotid artery 10 days after balloon catheter injury (A and B). 14 days after injury (C and D), or in uninjured carotid arteries (E and F). ni, neointima; m, media; a, adventitia. Arrows indicate the internal and external elastic laminae.
Fig. 2. Expression of distinctly spliced FGFR-2 and FGFR-3 isoforms in neointimal SMCs 10 days after injury. A, schematic structure of “prototypical” FGFR showing regions to which primers for RT-PCR were made to determine the extracellular ligand-binding domains of the FGFR-2 and FGFR-3 splice isoforms expressed by neointimal SMCs. Predicted sizes of the cDNA fragments amplified by each primer pair are shown. SP, signal peptide; AB, acidic box; HB, heparin-binding domain; I, II and IIIb/c, immunoglobulin-like domains and the presence of either the IIIb or IIIc exon in the COOH terminus of Ig-like domain III; TM, transmembrane domain. B, RT-PCR analysis of FGFR-2 mRNA expressed within the neointima (S represents dX174, digested with HaeIII, as size standard). Amplification with the Region A primers is shown in lane 1, whereas lane 2 shows amplification with the Region B-TM primers. Digestion of the 562-bp Region B-TM fragment with the IIIc-specific endonuclease HpaI is shown in lane 3. C, predicted structures of the predominant FGFR-2 isoforms expressed. D, RT-PCR analysis of FGFR-3 mRNA expressed within the neointima. Amplifications with primers encompassing Region A, Region A-IIIb, and Region A-IIIc are shown in lanes 1, 2, and 3, respectively, whereas lane 4 shows the amplification with Region B-TM primers. E, predicted structures of the predominant FGFR-3 isoforms expressed.

2B, lane 1); an additional two minor FGFR-2 cDNA species running below the full-length fragment were determined by diagnostic restriction endonuclease digestion (data not shown) to arise from aberrant splicing events within the Ig-like domain I exon. The two predominant amplification products were in the size range 250–350 bp and were characterized further by nucleotide sequencing, which confirmed that the larger fragment was 332 bp in length and was the consequence of splicing events that resulted in the deletion of the 264-bp exon encoding Ig-like domain I; the smaller fragment was 254 bp in length and had arisen from the deletion of both the Ig-like domain I exon and the subsequent 78-bp exon encoding the acidic box motif. Amplification using Region B-TM primers yielded the expected product (562 bp), which was completely cleaved with HpaI (Fig. 2B, lane 3), a restriction endonuclease specific for the IIIc exon, therefore indicating the presence of only the IIIc exon in this cDNA fragment. Thus, SMCs within the growing neointima of healing arteries express predominantly two FGFR-2 isoforms that lack the Ig-like domain I (“FGFR-2β”) forms, both of which contain exon IIIc and differ only by the presence or absence of the acidic box motif (Fig. 2C). Such isoforms, designated “FGFR-2βIIIcAB” and “FGFR-2βIIIcAB,” respectively, have been shown in a number of cell types to interact strongly with FGF-1, FGF-4, and FGF-9, but not with FGFs such as FGF-7 (14, 17, 18).

Given these findings, it was therefore of interest to also delineate the FGFR-3 isoforms expressed by the neointimal SMCs by using the PCR primers depicted in Fig. 2A. Amplification with Region A primers, which encompass the extracellular region that includes Ig-like domains I and II (nucleotides 6–452 of rat cDNA), yielded the predicted sized product of 447 bp (Fig. 2D, lane 1), indicating the presence of the exon encoding for Ig-like domain I. Additional amplification of the extracellular region by using the sense primer of Region A in combination with an antisense primer specific for either the IIIb exon (Region A-IIIb, nucleotides 6–981 of rat cDNA) or the IIIc exon (Region A-IIIc, nucleotides 6–981 of rat cDNA) yielded the predicted sized fragments of 634 bp (Fig. 2D, lane 2) and 976 bp (Fig. 2D, lane 3), respectively, indicating the simultaneous expression of FGFR-3 isoforms that contain either the IIIb or IIIc exon in the carboxyl terminus of the third Ig-like domain. Confirmation that such isoforms were transmembrane-spanning was shown by the amplification with primers encompassing a 784-bp region (Region B-TM, nucleotides 913–1696 of rat cDNA) that surrounds the transmembrane domain, yielding the predicted sized product (Fig. 2D, lane 4). Thus, SMCs in the growing neointima, in addition to the two FGFR-2 isoforms, express two FGFR-3 isoforms that contain all three Ig-like domains and the acidic box “FGFR-3α” forms and that differ from each other only by the alternate presence of either the IIIb or the IIIc exon and are designated as either FGFR-3αIIIbAB or FGFR-3αIIIcAB, respectively (Fig. 2E). Previously, it has been demonstrated in transfection studies that expression of either FGFR-3 isoform into L6 or BaF3 cells markedly increases their mitogenic responses to FGFs, including FGF-1 and FGF-9 (17, 28). Therefore, the FGFR-2/FGFR-3 isoforms expressed in the SMCs of the growing neointima in the injured carotid artery possess the ability to contribute to neointimal SMC proliferation, depending on the co-expression profile of the FGF ligands with which they are known to interact.

Dominant Negative Mutant FGFRs and the Inhibition of Neointimal SMC Proliferation—To determine whether one or more of these FGFRs contributed to neointimal SMC proliferation, we next examined the extent to which dominant negative mutant forms of the receptors could inhibit such proliferation. Previously, overexpression of dominant negative mutant
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FGFRs that contain the extracellular domains but are devoid of the intracellular tyrosine kinase domain have been used to differentiate the roles of endogenous FGFR-1, FGFR-2, and FGFR-3 in lens fiber differentiation and the development of diabetes (29, 30). Initially, we infected cultured neointimal SMCs with recombinant adenoviruses expressing cDNAs encoding DN-mutant forms of FGFR-2βIIIcAB, FGFR-3αIIIbAB, and FGFR-3αIIIcAB to confirm their expression as transmembrane-anchored proteins of the appropriate size. Two days after infection with the DN-FGFR-2βIIIcAB adenovirus (Fig. 3A, lane 2), SMCs expressed a minor peptide band of 37 kDa, which is the predicted size of the receptor protein in the absence of any significant glycosaminoglycan modification, and an intense broad band of ~55–80 kDa, which is consistent with this receptor isoform undergoing extensive post-translational modification by glycosaminoglycans (19). In contrast, infection of the SMCs with either the DN-FGFR-3αIIIbAB adenovirus (Fig. 3A, lane 4) or the DN-FGFR-3αIIIcAB adenovirus (Fig. 3A, lane 6) resulted in the predominant expression of the predicted 44-kDa protein with very little post-translational modification evident, which is consistent with findings that the presence of Ig-like domain I in these FGFR-3 isoforms largely abrogates any glycosaminoglycan modification occurring near the acidic box (19). To assess the ability of the DN-FGFR adenoviruses to inhibit neointimal SMC proliferation, carotid arteries injured 7 days earlier were exposed to each adenovirus (100 μl of a 5 × 10^8 plaque-forming unit solution), and 72 h later the effects on DNA synthesis were assessed by immunohistochemical detection and quantification of BrdUrd incorporation (percentage of BrdUrd-positive neointimal SMCs). In such experiments, the DN-FGFR-2βIIIcAB adenovirus was able to inhibit neointimal SMC proliferation by 40% relative to the level of proliferation present after infection with the control, a GFP-expressing adenovirus (p < 0.01; Fig. 3, B and C). Thus, as in NIH 3T3 cells (19), the endogenously expressed FGFR-2βIIIcAB isoform appears to be a potent stimulator of neointimal SMC proliferation. In contrast, infection of the vessels with an adenovirus expressing DN-FGFR-3αIIIcAB did not affect neointimal SMC proliferation (Fig. 3B) and, although infection with an adenovirus expressing DN-FGFR-3αIIIbAB reduced neointimal SMC proliferation by ~10%, this effect was not statistically significant (p > 0.05; Fig. 3B). Using Western blotting, we also assessed the level of DN-FGFR proteins and GFP (the expression of which is driven independently by a cytomegalovirus promoter in all four adenoviruses) (25) and confirmed that equivalent adenoviral expression occurred within arteries in each of the four treatment groups (Fig. 3B, insets).
Expression of Fibroblast Growth Factors during Neointimal SMC Proliferation—Because FGFR-2βIIIc and FGFR-3αIIb are known to exhibit a high affinity for FGF-1 and FGF-9 with a resultant stimulation of cellular proliferation (17, 28), we next examined the potential for these two FGF ligands to contribute to the neointimal SMC proliferation in healing arteries. First, we examined by immunohistochemistry the expression of FGF-1, which does not possess a hydrophobic signal sequence and is not normally secreted by cells (31). Ten days after the injury, when up to half of the SMCs in the neointima are proliferating, FGF-1 is barely detectable in this region of the healing artery, contrasting with its high concentration in the relatively quiescent medial region (Fig. 4A). In contrast, FGF-9 is highly expressed in the neointima at this time after injury, being diffusely dispersed throughout this region of the vessel (Fig. 4B) but barely detectable in uninjured arteries (Fig. 4C). Neointimal FGF-9 expression was confirmed both by RT-PCR (Fig. 4D, section a) and Western blot analyses (Fig. 4D, section b) of RNA and protein extracted from neointimal tissue obtained 10 days after injury. To determine the relationship between neointimal SMC proliferation and FGF-9 expression in the healing arteries, we carried out in situ RT-PCR for FGF-9 mRNA in arterial cross-sections collected 14 days after the injury. This analysis showed FGF-9 mRNA expression to be clearly apparent in SMCs adjacent to the lumen of the vessel (Fig. 5A; specificity of amplification/staining is shown by comparison to an equivalent cross-section not subjected to reverse transcription (Fig. 5B)). Notably, FGF-9 expression was observed after exposing these SMCs to FGF-7, which is not a mitogen for these neointimal SMCs. Initially, we examined its ability to stimulate mitogenesis in quiescent cultured SMCs prepared from the neointimas of carotid arteries injured 10 days earlier. Exposing the SMCs to FGF-9 resulted in a 30-40% increase in [3H]thymidine incorporation levels observed after exposing these SMCs to FGF-7, which is consistent with our finding that the high affinity FGFR for this ligand, FGFR-2βIIIb, was not appreciably expressed by neointima.
mal SMCs (see Fig. 2, B and C). Activation of the ERK 1/2 pathway has been shown to play a major role in neointimal SMC proliferation following arterial injury (32), so we next examined the ability of FGF-9 to activate these signaling proteins. In such experiments, FGF-9 induced a rapid, marked elevation in ERK 1/2 phosphorylation, which remained somewhat increased for at least 16 h (Fig. 5E, p-ERK row), suggesting an involvement of FGF2/IIIc (19); additional probing for levels of total ERK (Fig. 5E, ERK row) confirmed equivalent protein loading of all samples. To confirm the involvement of this receptor, the SMCs were exposed to an adenovirus expressing the DN-FGFR-2βIIIcAB for 48 h before the addition of FGF-9 and an assessment of ERK 1/2 activation. In such experiments, FGF-9 was unable to elicit ERK 1/2 activation after 10 min of exposure to the SMCs (Fig. 5F, p-ERK row, left) compared with the normal activation in the SMCs not pre-exposed to the DN-FGFR-2βIIIcAB adenovirus (Fig. 5F, p-ERK row, right). Under both sets of experimental conditions the SMCs remained equally responsive to platelet-derived growth factor-BB, which elevated ERK 1/2 phosphorylation to a similar extent in the presence (Fig. 5F, left section of row labeled p-ERK) or absence (Fig. 5F, p-ERK row, right) of DN-FGFR-2βIIIcAB; additional probing for levels of total ERK (ERK row) and DN-FGFR-2βIIIcAB (FLAG row) confirmed, respectively, equivalent protein loading of all samples and equivalent expression of the DN-FGFR-2βIIIcAB adenovirus within the infected SMCs. Taken together, these findings provide further support for the interaction between FGF-9 and endogenous FGFR-2βIIIcAB to play a major role in the proliferation of neointimal SMCs subsequent to arterial injury.

Because FGF-9 was mitogenic for neointimal SMCs in vitro, we next determined its contribution to peak arterial SMC proliferation in the injured arteries. Eight days subsequent to the initial balloon catheter injury, we delivered locally a 20 μM solution of a phosphorothioate-modified antisense FGF-9 oligonucleotide, which was designed toward the 5′-end of the mRNA (bases 170–184 of the rat cDNA) to most effectively disrupt the translation of the FGF-9 protein, and assessed neointimal SMC proliferation 48 h later. In such experiments, the antisense oligonucleotide inhibited neointimal SMC proliferation by ~52% (Fig. 6A), whereas identical administration of the corresponding phosphorothioate-modified sense oligonucleotide did not inhibit neointimal SMC proliferation (Fig. 6A). Immunohistochemical assessment of FGF-9 protein levels in arteries administered either the sense oligonucleotide (Fig. 6B) or the antisense oligonucleotide (Fig. 6C) demonstrated a marked reduction in the amount of FGF-9 protein within the neointima of the antisense oligonucleotide-treated arteries, providing further support for a major role of this FGF in neointimal SMC proliferation.

**DISCUSSION**

The identity of the growth factor/growth factor receptor signaling systems responsible for the proliferation of SMCs within the arterial intima/neointima has remained largely unknown. An involvement of FGF-FGFR signaling in intimal/neointimal SMC proliferation has been suggested, based on studies demonstrating the presence of FGFR-1 in healing arteries (11) and the expression of FGFR-1, FGFR-2, and FGFR-3 in atherosclerotic arteries (12, 13). However, although FGFR-1 has been shown to be involved in the early medial SMC proliferation stimulated by arterial injury, it appears not to be involved in neointimal SMC proliferation (11). To date, no study has determined the role that FGFR-2 and FGFR-3 play in this process or identified the FGF ligands responsible for neointimal SMC proliferation.

Here, we critically tested the contribution of FGFR-2, FGFR-3, and FGF-9 to neointimal SMC proliferation. We found that balloon catheter injury to the rat carotid artery stimulated the expression of two distinctively spliced FGFR-2βIIIc isoforms, differing only by the presence/absence of the acidic box, as well as two distinctively spliced FGFR-3α isoforms containing the acidic box and differing from each other by the presence of either the IIIb or the IIIc exon. Using post-injury arterial administration of recombinant adenoviruses expressing dominant negative mutant forms of these FGFRs to assess the roles of the endogenous FGFR isoforms in neointimal SMC proliferation, we found that DN-FGFR-2βIIIcAB significantly inhibited such proliferation, whereas the DN-FGFR-3α forms had little effect. We also found abundant expression in the neointima of FGF-9, an FGF ligand capable of binding to the FGFR-2/FGFR-3 isoforms. FGF-9 markedly stimulated in vitro the proliferation of cultured neointimal SMCs and the activation of ERK 1/2, the latter effects of which were abrogated by expression of the DN-FGFR-2βIIIcAB adenovirus; finally, in vivo administration of antisense FGF-9 oligonucleotides to in-
jured arteries significantly inhibited neointimal SMC proliferation, concomitant with a reduction in FGF-9 protein levels.

In a number of cell systems, FGF-9 appears to act as an autocrine/paracrine growth factor and has been shown to stimulate endometrial stromal cell proliferation (33) and lomocytation of human teratocarcinoma cells (34). It is actively secreted, and this feature distinguishes it from a number of other FGFs that are localized intracellularly and require cell damage for release (31). In *vivo*, FGF-9 exhibits cell type-specific effects. It has been implicated in sex determination, stimulation of mesenchymal cell proliferation, mesonephric cell migration, and Ser-toli cell differentiation in embryonic testis (35, 36). It supports survival of cholinergic neurons (37), and its overexpression disturbs skeletal development and linear bone growth (38). Our observation that FGF-9 signaling has been reported to sustain both autophosphorylation and subsequent phosphorylation of the receptor is consistent with the inability of these forms to significantly contribute to neointimal SMC proliferation (39). This interaction leads to a sustained activation of the ERK 1/2 pathway and the rapid proliferation of neointimal SMCs. Thus, targeting of this pathway may represent a useful approach aimed at inhibiting proliferative vascular diseases and related pathological processes involving neointimal SMC proliferation and accumulation.

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