Rad GTPase Attenuates Vascular Lesion Formation by Inhibition of Vascular Smooth Muscle Cell Migration

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Background—Rad (Ras associated with diabetes) GTPase is a prototypic member of a new subfamily of Ras-related GTPases with unique structural features, although its physiological role remains largely unknown. In the present study, we characterized the Rad function in vascular smooth muscle cells (VSMCs) and the influence of adenovirus-mediated Rad (Ad-Rad) gene delivery on vascular remodeling after experimental angioplasty.

Methods and Results—We documented for the first time that neointimal formation using balloon-injured rat carotid arteries was associated with a significant increase in Rad expression as determined by immunohistochemistry and quantitative real-time reverse-transcriptase polymerase chain reaction. The levels of Rad expression in VSMCs were highly induced by platelet-derived growth factor and tumor necrosis factor-α. Morphometric analyses 14 days after injury revealed significantly diminished neointimal formation in the Ad-Rad–treated carotid arteries compared with Ad-GFP or PBS controls, whereas the mutated form of Rad GTPase, which can bind GDP but not GTP, increased neointimal formation. Overexpression of Rad significantly inhibited the attachment and migration of VSMCs. In addition, Rad expression dramatically reduced the formation of focal contacts and stress fibers in VSMCs by blocking the Rho/ROK signaling pathway.

Conclusions—Our data clearly identified Rad GTPase as a novel and critical mediator that inhibits vascular lesion formation. Manipulation of the Rad signaling pathway may provide new therapeutic approaches that will limit vascular pathological remodeling. (Circulation. 2005;111:1071-1077.)

Key Words: atherosclerosis ■ cell movement ■ cells, vascular smooth muscle ■ monomeric GTP-binding proteins ■ restenosis

Activation of vascular smooth muscle cells (VSMCs) is a key event in vascular proliferative diseases such as primary atherosclerosis, postangioplasty restenosis, and bypass graft failure.1,2 The vascular injury that provokes VSMC proliferation, migration, and apoptosis is believed to contribute to the vascular lesion formation that occurs after balloon angioplasty and hence is of particular importance in the development of lesions within stents and bypass grafts.3,4 To date, the endogenous regulators that control VSMC migration, proliferation, and apoptosis remain to be fully defined. Emerging data suggest that small G proteins (also called GTPases) are implicated in the regulation of VSMC contraction, proliferation, migration, and apoptosis, as well as endothelial functions.5 Targeting small G proteins and their downstream signaling pathway may provide novel therapeutic approaches for the treatment of cardiovascular disorders such as atherosclerosis, restenosis, hypertension, and vasospasm.5

Rad (Ras associated with diabetes) is the prototypic member of a newly emerged Ras-related GTPase family with several unique characteristics, including Rad, Gem/Kir, Rem, Rem2, and Ges.6–11 It was initially identified by subtraction cloning as an mRNA overexpressed in skeletal muscle of a subset of humans with type 2 diabetes and normally is highly expressed in the heart and lung.6 Rad interacts with calmodulin, calmodulin-dependent protein kinase II,12 and β-tropomyosin.13 These interactions are enhanced by an increase in calcium influx and favor the inactive GDP-bound form of Rad.13 The exact function of Rad in normal tissues is still unknown, however.

In the present study, we have documented that Rad is a critical mediator that inhibits vascular lesion formation...
through suppression of VSMC migration, suggesting that manipulation of Rad signaling pathway may limit pathological remodeling.

Methods

Animals
Male Sprague-Dawley (300 to 350 g) rats were used in the balloon injury model (Harlan, Indianapolis, Ind). All animal experiments were performed according to National Institutes of Health guidelines and were approved by the Animal Care and Use Committee at the Morehouse School of Medicine.

Cell Culture
The rat embryonic aortic SMC line (A7r5) and rat primary aortic SMCs were purchased from the American Type Culture Collection and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (vol/vol) FBS in a 5% CO₂ humidified atmosphere at 37°C. A7r5-based stable cell lines were generated by stable transduction with puromycin resistance vector (pBabe) or pBabe containing the human wild-type or the dominant negative mutant (RadS105N) Rad cDNAs. Human aortic SMCs (HASMCs) purchased from Cambrex were used for experiments at passages 4 through 6 as previously described.

Construction of Adenovirus
Recombinant adenoviruses containing the human Rad cDNA or its dominant negative mutant (RadS105N) cDNA were prepared by the AdEasy system as previously described. The GFP adenovirus (Ad-GFP) was used as the control in this study.

Rat Carotid Artery Injury Model and Adenoviral Gene Transfer
The rat carotid artery (CA) balloon injury model was based on a model described by Clowes et al. The protocol used for treating rat CAs with an adenovirus has been previously described. The injured CA was washed with PBS and incubated with 25 μL PBS or adenovirus (5×10⁹ pfu/mL) carrying GFP, Rad, or RadS105N, respectively, for 20 minutes. The balloon-injured segment of the artery from the proximal edge of the omohyoid muscle to the carotid bifurcation was perfused with saline and dissected. The tissue was then fixed with 4% paraformaldehyde and embedded in paraffin. Subsequent morphometric analyses were performed in a double-blinded manner. To monitor the efficiency of adenoviral gene delivery, we performed an additional experiment using adenovirus expressing LacZ gene (Ad-LacZ). Rat CAs from Ad-LacZ infected derived stable expression of Rad or mutant Rad, RadS105N, cells (5×10⁸) were loaded into the upper volume of the Boyden chambers. Six hours later, nonmigrated cells were removed with a cotton swab, and the migrated cells were fixed with methanol for 10 minutes and stained with hematoxylin. Cell migration was quantified by blind counting of the migrated cells on the lower surface of the membrane of ≥5 fields per chamber under microscope. For the scratch wound motility assay, a linear scratch (~4 mm wide) was done with a cell lifter across the diameter of the well and rinsed with PBS. Cells were kept in serum-free medium for 48 hours. For each well, pictures were taken on a dissection microscope at a magnification of ×40 at 2 time points, 0 and 48 hours after scratch. The migration distance was calculated by the difference of the gap at the 2 time points.

Northern Blot and Western Blot Analyses
Northern blot and Western blot analyses were performed as previously described. A rabbit anti-Rad polyclonal antibody generated by Kahn’s group was used in this study.

Immunofluorescent Staining
Exponentially growing cells were plated on glass coverslips in 6-well cell culture plates and incubated overnight at normal cell growth conditions. The immunofluorescent staining was performed as previously described.

Quantitative Real-Time Reverse-Transcriptase Polymerase Chain Reaction
Quantitative real-time reverse-transcriptase polymerase chain reaction (QRT-PCR) was carried out with a LightCycler thermocycler and an SYBR green I kit (Roche Diagnostics Corp) according to the manufacturers’ recommendations. The relative mRNA levels of Rad normalized by GAPDH were evaluated with the following specific primers: Rad-up, 5'-GGGCCGCTAGTAGCTCGCAACAG-3'; Rad-down, 5'-CTTCCCTCGAGAGCATCCACAT-3'. Cycle numbers obtained at the log-linear phase of the reaction were plotted against a standard curve prepared with serially diluted control samples.

Immunoprecipitation and Rho Kinase Assay
A7r5 cells were transfected with Flag-tagged Rad or cotransfected Flag-Rad with ROKα or Rho A by Lipofectamine 2000 (Invitrogen). At 24 hours after transfection, the cells were scraped off and lysed in cell lysis buffer as previously described. The cell lysates were subjected to immunoprecipitation with either anti-ROKα (BD Bioscience) or anti-Flag (Sigma) monoclonal antibody. The supernatant and bound fractions were used for immunoblot analysis. For Rho kinase assay, A7r5 cells were transfected with myc-tagged ROKα or cotransfection ROKα with Rad. Equal amounts of lysate proteins were subjected to immunoprecipitation with anti-myc monoclonal antibody (clone 4A6, Upstate). Immunoprecipitates were further washed twice with kinase-assay buffer and finally resuspended in 50 μL of kinase-assay buffer containing 10 μmol/L ATP, 1.5 μg recombinant myosin phosphatase target subunit 1 (MYPT1, Upstate), which is a specific substrate of Rho-kinase, and 1 μCi γ-³²P-ATP (PerkinElmer). Samples were incubated for 30 minutes at 30°C with agitation, terminated by addition of loading buffer, run on 10% SDS-PAGE, and then dried. Recombinant rat ROK protein (Upstate) served as a positive control. Relative kinase activity was scanned with the Typhoon Phosphoimager.

Statistical Analysis
All data were evaluated with a 2-tailed, unpaired Student t test or compared by 1-way ANOVA followed by Fisher t test and are
expressed as mean±SD. A value of $P<0.05$ was considered statistically significant.

Results

Rad Expression Is Increased During Vascular Lesion Formation

As an initial step to define the role of Rad in vasculature, we compared Rad expression levels between normal and injured vessels using balloon-injured rat CAs. Our results demonstrated that neointimal formation in this model was associated with an increase in Rad expression as determined by immunohistochemistry (Figure 1A). Rad staining was negligible in the control of uninjured arteries. In contrast, strong expression of Rad was observed in the developing neointima and media. It reached its peak at 2 weeks and remained elevated 4 weeks after vascular injury. To further determine the expression pattern of Rad after vascular injury, we performed Western blot and QRTPCR experiments using protein and RNA samples extracted from balloon-injured rat CAs at different time points. The levels of Rad mRNA expression were upregulated by $\approx 3$-fold at 4 hours, reached a maximal 9-fold increase at 8 hours, but returned to baseline after 1 day of balloon injury (Figure 1B). Consistent with results from Figure 1A, Rad mRNA expression levels were upregulated again by 4-fold at 2 weeks and remained at the elevated level 4 weeks after balloon injury (Figure 1B). A similar expression pattern of Rad protein was observed in balloon-injured CAs by Western blot analysis (Figure 1C). Taken together, there are 2 peaks (at 8 hours and 2 weeks) of Rad gene expression after vascular injury. It will be of interest to study molecular mechanisms of this biphasic induction.

Because growth factors and cytokines such as platelet-derived growth factor (PDGF) and tumor necrosis factor-α (TNFα) play important roles in vascular lesion formation, we postulated that these factors might regulate Rad gene expression in VSMCs. As shown in Figure 1D, both PDGF (10 ng/mL) and TNFα (10 ng/mL) markedly induced Rad mRNA expression in HASMCs, which started after 15 minutes of stimulation and peaked at 2 hours by Northern blot analysis. In addition, we documented that PDGF dose dependently induced Rad expression by Northern and Western blot analyses (Figure 1E). Similar results were obtained using rat aortic SMCs (data not shown). Taken together, our results suggest that Rad may be a novel mediator during vascular lesion formation.

Rad Suppresses Neointimal Formation In Vivo

To investigate the role of Rad in neointimal formation in vivo, we used a highly characterized balloon-injured rat CA model and adenoviral gene transfer technology. Recombinant adenoviruses containing GFP (Ad-GFP), Rad (Ad-Rad), and dominant negative Rad mutant (Ad-RadS105N) were successfully generated for this study (Figure 2A). Successful adenoviral infections were demonstrated in balloon-injured rat CAs treated with Ad-GFP or Ad-LacZ by Western blot analysis or X-Gal staining. As shown in Figure 2B and 2C, the GFP expression levels were detected at day 1, reached the maximum level at day 4, and remained high until day 7 after adenoviral infection. Strong LacZ expression was observed in the vessels 4 days after being treated with Ad-LacZ. Most infected cells were medial VSMCs, and $\sim 40\%$ of the cells were infected ($n=6$). Intriguingly, we found that balloon-injured CAs transduced with Ad-Rad exhibited a marked reduction in neointimal formation 14 days after injury compared with arteries transduced with Ad-GFP (Figure 2D). Morphometric analysis of elastic-stained sections showed that expression of Ad-Rad suppressed neointimal formation by 57% ($0.028\pm0.005$ versus $0.063\pm0.012$ mm$^2$; $P<0.01$;
RadS105N revealed an increase in neointimal formation 14 days after vascular injury compared with those transduced with Ad-GFP or PBS (Figure 2D). Morphometric analysis of elastic-stained sections revealed that expression of Ad-RadS105N increased neointimal formation by 38% (0.097±0.020 versus 0.063±0.012 mm²; P<0.05; n=8) compared with Ad-GFP–transduced vessels. In addition, there was no significant difference between Ad-GFP– and PBS-treated groups (Figure 2E). Furthermore, the similar effects of Ad-Rad and Ad-RadS105N were found at an earlier time point, day 9 after balloon injury (data not shown). Taken together, our data suggest that Rad inhibits vascular lesion formation.

**Rad Inhibits VSMC Attachment and Migration In Vitro**

VSMC migration is thought to play a central role in atherogenesis and restenosis. VSMC migration from the media into the intimal surface of the blood vessels is an important step during neointimal formation after vascular injury. To delineate the mechanisms by which Rad inhibits intimal hyperplasia, we used an in vitro system to evaluate the effects of Rad on VSMC attachment and migration. A7r5 cells were stably transduced with or without the pBabe retroviral vector, the vector containing wild-type Rad, or the vector containing RadS105N cDNA. These cell lines were designated as A7r5, Babe, Rad, and RadS105N cells. Western analysis showed that the levels of Rad protein were highly expressed in Rad and RadS105N cell lines (Figure 3A). To define whether Rad affects VSMC attachment, ~1.5×10⁴ cells from each cell line were seeded in a fibronectin-coated 6-well plate and cultured under normal conditions. At different times as indicated in Figure 3B, the attached cells were trypsinized and counted. The results demonstrated that Rad significantly inhibited cell attachment and RadS105N increased cell attachments compared with A7r5 and Babe cell lines (P<0.05; n=6). To further determine whether Rad regulates VSMC migration, we used 2 independent approaches: a modified Boyden assay¹⁹ and a monolayer scratch wound assay.²¹ Both approaches clearly showed that Rad significantly inhibited cell migration and RadS105N increased its mobility (Figure 3C and 3D). Similar results were found in HASMCs infected with Ad-GFP, Ad-Rad, or Ad-RadS105N (data not shown). Thus, our data demonstrate that Rad expression inhibits VSMC attachment and migration in vitro.

**Rad Inhibits the Formation of Focal Adhesions and Actin Stress Fibers**

To determine the mechanism of Rad-inhibited VSMC migration, we examined whether Rad affects cytoskeletal organization. Cells were seeded on fibronectin-coated slides and cultured under normal conditions for 6 hours and then fixed. The focal adhesion contacts and actin stress fibers were labeled by anti-vinculin and anti–α-actin, followed by FITC-conjugated secondary antibody. As shown in Figure 4A, overexpression of Rad in A7r5 cells abolished and RadS105N enhanced focal adhesion contacts and the organization of smooth muscle α-actin–containing

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n=8) without altering medial wall dimensions compared with Ad-GFP–transduced vessels (Figure 2E).

To verify whether the endogenously expressed Rad negatively regulates neointimal formation after vascular injury, we constructed an adenovirus containing a putative dominant negative mutant with a Ser-to-Asn mutation at position 105, which is analogous to an S17N mutation in Ras and devoid of GTP binding but still binds GDP.¹⁴ As previously reported, the mutated form of Rad GTPase (RadS105N) can function as a dominant negative mutant.²⁵ Arteries transduced with Ad-RadS105N revealed an increase in neointimal formation 14...
stress fibers. In addition, overexpression of Rad in A7r5 cells by adenoviral infection also eliminated focal adhesion contacts, which was similar to the inhibition of ROK signaling pathway by Y27632 (10 μmol/L; Calbiochem), a specific ROK inhibitor (Figure 4B). This series of experiments document that Rad inhibits focal adhesion and stress fiber organization by reducing VSMC attachment and migration.

Rad Negatively Regulates Rho/ROK Signaling Pathway

It has been well documented that cytoskeletal organization is regulated by several signaling pathways, including pathways involving integrin/FAK, PI3-kinase/Akt, and Rho/ROK. To further explore the mechanisms by which Rad regulates cytoskeletal organization, we investigated the effect of Rad on the above signaling pathways.

Using Western blot analyses, we documented that Rad could not significantly affect the phosphorylation of GSK3, Akt, and FAK (data not shown). Consistent with a previous report that Rad bound ROKα in neuroblastoma cells,22 we documented that Rad could physically interact with ROKα but not with Rho A in VSMCs by immunoprecipitation assays (Figure 5A). To examine whether Rad affects ROKα activity, we performed the kinase assay, followed by immunoprecipitation. As shown in Figure 5B, we demonstrated that cotransfection of Rad with ROKα vectors in A7r5 cells significantly inhibited ROKα activity (42% reduction) compared with those only transfected with ROKα. However, the Rad dominant negative mutant (RadS105N) lost the capability to inhibit ROKα kinase activity, demonstrating that Rad-inhibiting ROK activity is GTP dependent. Taken together, our data suggest that Rad reduces VSMC migration, at least partially, by inhibiting the ROK singling pathway.

Discussion

To date, >100 GTPases have been identified and exert a wide spectrum of functions, including regulation of gene expression; cell proliferation, migration, and apoptosis; cytoskeletal rearrangement; and intracellular vesicle trafficking.26 Although the structural features of the Rad GTPase subfamily have been known for many years, the physiological role of the individual protein is not well defined. Data in the present study documented for the first time that Rad GTPase plays an important role in the reduction of neointimal formation after angioplasty.

By using the highly characterized balloon-injured rat CA model and adenoviral gene transfer technology, we found that overexpression of Rad GTPase dramatically reduced vascular lesion formation after balloon injury. This action of Rad GTPase on neointimal formation is dependent on GTP loading. RadS105N, a point mutation analogous to RasS17N that is devoid of GTP binding but still binds GDP,14 not only lost the inhibitory action on neointimal formation but significantly increased neointimal formation, suggesting that the endogenous Rad GTPase is a negative regulator in vascular lesion formation.

The VSMC accumulation in the neointima is theoretically the sum of cell migration and proliferation and...
apoptotic cell death; an alteration in any of these events can affect vascular lesion formation. The present study demonstrated that Rad dramatically inhibited VSMC migration, which may account for Rad-reduced vascular lesion formation. Focal adhesion formation and cytoskeletal organization are the key processes in cell locomotion and migration. It was well defined that Rad was localized in cytoskeleton and interacted with calmodulin and β-tropomyosin, suggesting that Rad may play a role in cytoskeleton organization. In addition, it was reported that Gem and Rad affected the formation of stress fibers and focal adhesion contacts in neuroblastoma cells. In the present study, we documented that overexpression of Rad by either transient or stable transfection in VSMCs abolished focal adhesion and disassembled actin stress fibers.

It is well established that several signaling pathways, including integrins/FAK, PI3-kinase/Akt, and Rho/ROK, play important roles in the regulation of cytoskeleton organization and cell migration. In the present study, we found that overexpression of Rad by either transient or stable transfection in VSMCs abolished focal adhesion and disassembled actin stress fibers.

In summary, we have identified Rad as a critical negative regulator of vascular lesion formation. It has been well established that vascular injury can activate a set of gene expressions such as PDGF and TNFα. The products of these genes trigger several signal events, promote VSMC proliferation and migration, and regulate VSMC apoptosis, which eventually leads to neointimal formation. From the present results, it is tempting to speculate that the growth factors and cytokines-induced Rad expression associated with vascular injury may represent a novel negative feedback mechanism that prevents VSMC migration from overresponse to injury-based stimuli. Targeting Rad GTPase signaling pathways may provide new therapeutic approaches for the treatment of occlusive vascular diseases such as primary atherosclerosis, restenosis, and vein graft failure.
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