Activated Forkhead Transcription Factor Inhibits Neointimal Hyperplasia After Angioplasty Through Induction of p27

Kyung-Woo Park, Dae-Hee Kim, Hyun-Jung You, Jung-Ju Sir, Soo-In Jeon, Seock-Won Youn, Han-Mo Yang, Carsten Skurk, Young-Bae Park, Kenneth Walsh, Hyo-Soo Kim

Objective—We examined the effects of FKHRL1 (forkhead transcription factor in rhabdomyosarcoma like-1) overexpression on vascular smooth muscle cell (VSMC) proliferation, apoptosis, and cell cycle, in vitro, and the role of FKHRL1 and p27 in the pathophysiology of neointimal growth after balloon angioplasty, in vivo. Furthermore, we tested whether FKHRL1 overexpression can inhibit neointimal hyperplasia in a rat carotid artery model.

Methods and Results—Adenovirus expressing the constitutively active FKHRL1 (FKHRL1-TM; triple mutant) with 3 Akt phosphorylation sites mutated was transfected to subconfluent VSMCs. FKHRL1 overexpression in cultured VSMCs increased p27 expression, leading to G1 phase cell-cycle arrest and increased apoptosis. In vivo, the phosphorylation of FKHRL1 increased significantly 3 hours after balloon injury and decreased thereafter, with the subsequent downregulation of p27. Although the phosphorylation of FKHRL1 was greatest at 3 hours, the downregulation of p27 showed a temporal delay, only slightly starting to decrease after 3 hours and reaching a nadir at 72 hours after balloon injury. Gene transfer of FKHRL1-TM increased p27, decreased proliferation, and increased apoptosis of VSMCs, which resulted in a marked reduction in neointima formation (intima-to-media ratio: 0.31±0.13 versus 1.17±0.28, for FKHRL1-TM versus Adv-GFP; P<0.001).

Conclusion—Balloon angioplasty leads to the phosphorylation of FKHRL1 and decreased expression of p27, thereby promoting a proliferative phenotype in VSMCs in vitro and in vivo. This study reveals the importance of FKHRL1 in proliferation and viability of VSMCs and suggests that it may serve as a molecular target for interventions to reduce neointima formation after angioplasty. (Arterioscler Thromb Vasc Biol. 2005;25:742-747.)

Key Words: forkhead transcription factors ■ neointima ■ p27 ■ vascular smooth muscle cell

Despite the recent advances in strategies to prevent neointimal growth after angioplasty, it still remains the major limitation of percutaneous coronary interventions.1 Although the pathogenic mechanism of restenosis is complex, the proliferation and migration of vascular smooth muscle cells (VSMCs) after balloon injury seems to be a major factor in this process.2–4 Therefore, we along with many investigators have targeted VSMC proliferation and migration as a means to counter restenosis.5,6 FKHRL1 (forkhead transcription factor in rhabdomyosarcoma-like 1, FOXO3a) is a member of the forkhead transcription factor family, which is emerging as a key factor in pathways that regulate differentiation, metabolism, proliferation, and survival of cells.7 FKHRL1 is negatively regulated by the PI3K/Akt pathway.8–10 Phosphorylation of FKHRL1 by Akt leads to cytoplasmic retention and impairment of its nuclear transcriptional activity. Past studies have shown that the key actions of forkhead family transcription factors are cell-cycle arrest11–14 and apoptosis.9 Taken together, FKHRL1 may affect the cell cycle and apoptosis of VSMCs, and may serve as a therapeutic target to inhibit neointimal growth after angioplasty. In the present study, we examined the effects of FKHRL1 on VSMC survival and proliferation in vitro. We also investigated whether FKHRL1 plays a role in neointima formation after balloon injury, and we tested whether the cytotoxic and cystotic properties FKHRL1 can reduce neointimal hyperplasia after balloon injury in a rat carotid injury model.

Materials and Methods

Construction of Adenoviral Vectors Expressing Constitutively Active Triple Mutant

Detailed preparation procedures were previously described.11 The hemagglutinin-tagged human FKHRL1 triple mutant (TM: T32A/
FKHR1 and Apoptosis

Subconfluent rat SMCs were infected with adenoviruses (Adv-FKHR1-TM and Adv-GFP, respectively) at 50 multiplicities of infection (moi) for 48 hours, harvested with trypsin, fixed in cold 90% ethanol for 20 minutes, and then resuspended in staining buffer consisting of 1% RNase A, 20 μg/ml propidium iodide, and 0.01% NP40. DNA content was analyzed by flow cytometry on FL-2 channel and gating was set to exclude debris and cellular aggregates. For each analysis, 10,000 events were counted.

FKHR1 and Cell-Cycle Regulation

To test the effect of FKHR1 on cell cycle, rat SMCs were starved without serum and simultaneously infected with adenoviruses, Adv-FKHR1-TM and Adv-GFP, respectively, at 50 moi for 24 hours, and stimulated with 20% fetal bovine serum for 24 hours. After serum stimulation, cells were trypsinized, centrifuged at 1200 rpm for 5 minutes, washed with phosphate-buffered saline, and treated with RNase A (20 μg/ml). DNA was stained with propidium iodide (100 μg/ml) for 30 minutes at 4°C in the dark. DNA content of the cells was analyzed using a Becton Dickinson fluorescence-activated cell sorter Calibur flow cytometer (Becton Dickinson, San Jose, Calif). DNA histogram analysis was performed using ModFitLT software (Becton Dickinson). The percentage of each cell cycle phase was compared between 2 groups.

Immunoblot Assay

Cells were infected with adenoviral vector (Adv-FKHR1-TM or Adv-GFP) at 50 moi for 20 hours. Cells were washed in phosphate-buffered saline and harvested by scraping in 50 mmol/L Tris-HCl (pH 7.2), 250 mmol/L NaCl, 1% NP40, 0.05% SDS, 2 mmol/L EDTA, 0.5% deoxycholic acid, 10 mmol/L glycerophosphate, and 1 mmol/L phenyl methyl sulfonyl fluoride. The primary antibodies used were anti-total FKHR1 antibody (1:500 dilution; Upstate Biotechnologies), anti-HA (hemagglutinin; 1:4000; Roche), and anti-p27 (1:1000; BD Transduction Laboratories).

Measurements of phospho-FKHR1 and p27 in Carotid Arteries After Balloon Injury

A previously well-established rat carotid artery balloon injury model was used.8 The vessel was harvested at several serial points after balloon injury (3 hours, 24 hours, 72 hours, and 2 weeks) to examine the endogenous change in FKHR1 phosphorylation and p27 expression. Western blot analysis of vessel protein lysates using anti-phospho-FKHR1 antibody (1:100; Upstate Biotechnologies) and anti-p27 antibody (1:100; Santa Cruz) were performed. The serial change in the expression of phospho-FKHR1 and p27 were also confirmed by immunohistochemistry using phospho-FKHR1 antibody (1:50; Upstate Biotechnologies) and anti-p27 antibody (1:50; Santa Cruz).
FKHRL1 Overexpression Induces p27 Expression in Cultured VSMCs

Efficient transfection of constitutively active FKHRL1-TM gene was confirmed by detecting the expression of hemagglutinin, which was tagged onto the viral construct, as shown by Western blot analysis (Figure 1A; cells harvested 24 hours after gene delivery). The overexpression of FKHRL1-TM increased the expression of p27 compared with control. In addition, we observed the endogenous expression of FKHRL1 and its downstream effector molecule, p27, after balloon injury. Western blot of in vivo vessel samples at various time points showed that phosphorylation of FKHRL1 resulting in its inactivation occurs as soon as 3 hours after balloon injury, decreases at 24 hours, and then returns to baseline levels by day 3. The expression of p27, which we expected to be downregulated by phosphorylation of FKHRL1, showed a temporal delay in comparison to its up-stream transcription factor FKHRL1. Compared with phospho-FKHRL1, which was markedly increased at 3 hours, the expression of p27 was similar to baseline up to 3 hours after injury, slowly declining to its lowest expression at day 3 (Figure 2A). The changes observed with Western blot samples were well-correlated with the immunohistochemistry results of phospho-FKHRL1 (Figure 2B) and p27 (Figure 2C).

FKHRL1 Overexpression Decreases VSMC Viability: Effect of Cell-Cycle Arrest and Apoptosis

To investigate the effect of FKHRL1 overexpression on VSMC viability, serum-stimulated (fetal bovine serum 10%) rat VSMC cultures were transfected with either Adv-GFP or Adv-FKHRL1-TM at 50 moi, which results in >95% transfection efficiency. Cell viability was measured 24 and 48 hours after gene transfer using WST-1 assay. Overexpression of FKHRL1 significantly diminished cell viability at both time points (Figure 1B; *P<0.01, at both 24 and 48 hours).

Cell-cycle analysis using fluorescence-activated cell sorter analysis of the cell cycle showing a typical profile of G0/G1 cell-cycle arrest in the FKHRL1 group.Increased apoptosis after FKHRL1 overexpression as examined by fluorescence-activated cell sorter analysis and fluorescent microscopy. The construct of adenovirus-FKHRL1 also contains the GFP gene.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labeling Staining

Detection of apoptotic cells in vivo was also performed using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)31 method with minor modifications. Apoptotic cells were quantified by counting the percentage of TUNEL-positive cells against total nucleated cells in 4 different sections per tissue section (n=8).

Statistical Analysis

All data were presented as mean±SD. Comparisons between constitutively active FKHRL1 transfected and control group were performed using an unpaired 2-tailed t test, but the comparison of results from in vitro studies were performed using Mann–Whitney U test because of small numbers. Values of P<0.05 were considered significant. SPSS 11.0 software was used for all statistical calculations.

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age of hemagglutinin (which was tagged to the adenoviral vector) stained versus total medial and neointimal cells per arterial section, was 38.6%±10.7% (n=8). In the control GFP group, the expression of p27 was low at day 3 and increased thereafter at day 14. However, the gene transfer of constitutively active FKHRL1 resulted in a significant increase in p27 expression compared with GFP at day 3. Although the expression of hemagglutinin was faint at week 2, the higher expression of p27 in the FKHRL1-TM group was sustained (Figure 3A). Immunoprecipitation Western blotting of total FKHRL1 showed that endogenous total FKHRL1 expression did not change after balloon injury and was only significantly increased after FKHRL1-TM gene transfer (Figure 1A, available online at http://atvb.ahajournals.org). Furthermore, the cells transduced with the adenoviral vectors were confirmed as medial smooth muscle cells by double immunohistochemical staining (Figure 1B).

Constitutively Active FKHRL1-TM Gene Transfer Inhibits Proliferation and Increases Apoptosis of VSMCs After Balloon Injury

Immunohistochemical staining for PCNA was performed to examine the effects of the constitutively active FKHRL1 gene transfer on VSMC proliferation during neointimal formation after balloon injury. PCNA-positive cells were markedly reduced in the FKHRL1-TM transfected group (Figure 4A). The proliferative index (calculated as the fraction of positive cells among total nucleated cells) was significantly reduced in the FKHRL1-TM-transfected group at day 3 (0.16±0.03 versus 0.05±0.03 mm²; P<0.01), whereas there was no significant difference in medial area (0.17±0.03 versus 0.16±0.03 mm²; P=0.34) compared with the GFP-transfected group (Figure 3B and 3C). Although there were no significant differences in medial area, the cellularity of the media was significantly lower in the FKHRL1-TM-transfected group at day 3 (42.4±5.3 versus 28.9±6.1 cells/high-power field, for control versus FKHRL1-TM, n=8; P<0.01). This led to a significantly lower intima-to-media ratio (0.96±0.19 versus 0.35±0.17; P<0.01) and a greater lumen area (0.27±0.04 versus 0.35±0.06 mm²; P<0.01) compared with the GFP-transfected group (Figure 3B and 3C).
To determine the effect of constitutively active FKHR1 gene transfer on apoptosis after balloon injury in vivo, TUNEL staining (Figure 4A) and immunohistochemistry for active and cleaved caspase-3 (Figure IIA, available online at http://atvb.ahajournals.org) was performed at 3 days after balloon injury. The apoptotic activity of VSMCs was high in the GFP-transfected and FKHR1-TM-transfected group, although apoptotic activity was higher in the FKHR1-TM–transfected group (27.9±5.88 versus 46.5±4.05%, n=8, P<0.001 for GFP versus FKHR1-TM). Even at 2 weeks after balloon injury, when the apoptotic activity declined considerably, the apoptotic activity remained significantly higher in the FKHR1-TM–transfected group compared with the GFP-transfected group (7.3±1.11% versus 10.9±1.78%, n=8, P<0.001 for GFP versus FKHR1-TM) (Figure 4C). Furthermore, double immunohistochemistry showed that the cells undergoing apoptosis and thus positive for caspase 3 are smooth muscle cells (Figure IIB).

### Discussion

The process of neointima formation after balloon injury is complex, and elucidation of possible key molecules involved in the pathophysiology of neointimal hyperplasia may help in the efforts to reduce restenosis. In the present study, we report for the first time to our knowledge that FKHR1 and p27 play an important role in the response to balloon denudation injury and neointima formation in rats. In vitro, the overexpression of FKHR1 by constitutively active FKHR1-TM gene transfer resulted in increased apoptosis, induction of p27, and G1/S phase cell-cycle arrest of cultured VSMCs. In vivo, carotid balloon injury resulted in phosphorylation of FKHR1, which led to temporally delayed p27 downregulation and neointimal formation in rat carotid arteries. The importance of FKHR1 and p27 were confirmed by constitutively active FKHR1 gene transfer, which increased p27 expression and resulted in decreased neointimal hyperplasia after angioplasty.

FKHR1 (forkhead transcription factor in rhabdomyosarcoma like 1, FOXO3a) is a member of the forkhead transcription factor family, which has been shown to play an important role in cell-cycle arrest and apoptosis of various cells. In various cancer cell lines, endothelial cells, and VSMCs, forkhead transcription factors have been shown to induce G1 phase cell-cycle arrest. Recently, several studies demonstrated the regulation of p27 by forkhead transcription factors. Forkhead transcription factors were shown to mediate cell-cycle regulation by PKB through p27 but not p21 in cancer cell lines, and the inhibition of FKHR1 was reported to promote endothelial proliferation by downregulation of p27 in human endothelial cells. In VSMCs, inactivation of forkhead transcription factors by mechanical strain resulted in transcriptional downregulation of p27.

In addition, FHKRL1 has been shown to induce apoptosis in neuronal cell lines and fibroblasts by upregulation of Fas-ligand expression and activation of the death receptor pathway. However, in hematopoietic cells, the “intrinsic” mitochondrial pathway, rather than the “extrinsic” Fas-mediated pathway, was shown to promote apoptotic cell death after activation of FKHR1 signaling. In VSMCs, we previously showed that FKHR1 activates caspase 9, caspase 3, and c-Jun sequentially, and induces FasL to result in apoptosis under conditions of cellular stress. In the present study, overexpression of FKHR1 resulted in increased expression of p27 and decreased survival of cultured VSMCs, which was caused by increased apoptosis and cell-cycle arrest. Constitutively active FKHR1 gene transfer resulted in a marked increase in apoptosis and a typical profile of G0/G1 phase cell-cycle arrest (increase in cells in G1 phase and decrease in cells in S phase).

The p27kip1 is a cyclin-dependent protein kinase (CDK) inhibitor, which regulates the cell cycle via downregulation of CDK2 activity and repression of cyclin A transcription. CDK/cyclin holoenzymes facilitate the progression of the cell cycle and is negatively regulated by the interaction with specific CDK inhibitory proteins that cause cell-cycle arrest when overexpressed in transformed and nontransformed cell lines. Mice lacking the CDK inhibitor function of p27 display enhanced growth and multiple organ hyperplasia and overexpression of p27 in rat carotid arteries attenuated neointimal hyperplasia.

The sequential change in expression levels of p27 after vessel injury seems to be somewhat different among different species. Reis et al reported that p27 began to increase 48 hours after balloon injury and persisted through the fourth week in mouse femoral artery injury model, whereas in rat carotid arteries, p27 was already increased at 48 hours after balloon injury. In porcine artery injury model, p27 began to decrease after balloon injury, stayed at the bottom level from day 4 to day 14, and then began to increase. In the present study, we found that there is a temporal delay between the phosphorylation of FKHR1 and the consequent downregulation in p27 expression. Compared with FKHR1 phosphorylation, which is strongest at 3 hours after injury and declines to undetectable baseline levels by day 14, p27 expression only starts to decline slightly at 3 hours after balloon injury and reaches nadir at 72 hours, finally to be strongly re-expressed at day 14. The increase from baseline and decrease thereafter of phospho-FKHR1 coincides with the downregulation from baseline and upregulation thereafter of p27 except with a temporal delay. This sequential change is concordant with previous cell kinetic studies which showed that VSMC proliferation and migration starts early after vessel injury, is greatest at ~72 hours, and slowly declines to baseline levels by 2 weeks. Furthermore, we found that the gene transfer of constitutively active FKHR1 results in upregulation of p27 and significant inhibition of neointimal proliferation after angioplasty, suggesting the importance of FKHR1 phosphorylation and consequent downregulation of p27 in neointima formation. This is a novel finding that may have implications in future research. In the FKHR1-TM gene transfer group, FKHR1-TM was strongly expressed at day 3, whereas only faintly expressed at 2 weeks. Expression of p27, however, was moderate at day 3 and strong at 2 weeks, although its expression at each time point (3 days and 2 weeks) was significantly greater in the FKHR1-TM group.
than that of the control group. The disparity is probably because of the fact that after adenovirus-mediated gene transfer, the expression of the adenoviral transgene is highly expressed at 3 days and declines markedly to low levels by 2 to 3 weeks.30,31 Considering also that a significant portion of the gene-transfected smooth muscle cells underwent apoptosis, the faint expression of FKHRL1-TM at 2 weeks is not surprising. The high expression of p27, however, was more because of the endogenous restoration of p27 expression in VSMCs, as we showed in Figure 2A. As seen in Figure 2A, the rebound expression of p27 at 2 weeks after injury is even stronger than at baseline levels.

In conclusion, balloon angioplasty leads to the phosphorylation of FKHRL1 and downregulation of p27 resulting in a proliferative phenotype in VSMCs. FKHRL1 overexpression increases p27 expression, leading to G1/S phase cell-cycle arrest and increased apoptosis of VSMCs, thereby inhibiting neointimal hyperplasia. This study reveals the importance of FKHRL1 in proliferation and viability of VSMCs and suggests that it may serve as a target for interventions to inhibit neointima formation after angioplasty.

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