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UAP56 is an important mediator of Angiotensin II/Platelet Derived Growth Factor induced vascular smooth muscle cell DNA synthesis and proliferation

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Abstract

Angiotensin (Ang) II and platelet-derived growth factor (PDGF) are important mediators of pathologic vascular smooth muscle cell (VSMC) proliferation. Identifying downstream mediators of Ang II and PDGF signaling may provide insights for therapies to improve vascular proliferative diseases. We have previously demonstrated that breakpoint cluster region (Bcr) is an important mediator of Ang II/PDGF signaling in VSMC. We have recently reported that the DExD/H box protein UAP56 is an interacting partner of Bcr in regulating VSMC DNA synthesis. We hypothesized that UAP56 itself is an important regulator of VSMC proliferation. In this report we demonstrate that knockdown of UAP56 inhibits Ang II/PDGF induced VSMC DNA synthesis and proliferation, and inhibits E2F transcriptional activity. In addition, we demonstrate that UAP56 is present in the vessel wall of low-flow carotid arteries. These findings suggest that UAP56 is a regulator of VSMC proliferation and identify UAP56 as a target for preventing vascular proliferative disease.

Keywords

proliferation; DNA synthesis; UAP56; helicase

INTRODUCTION

Angiotensin II (Ang II) and platelet derived growth factor (PDGF) are important mediators of pathologic vascular smooth muscle cell (VSMC) proliferation seen in several cardiovascular diseases including hypertension and atherosclerosis [1–4]. Identifying downstream mediators of Ang II and PDGF signaling may provide insights for therapies to improve vascular proliferative diseases.

We have previously reported that breakpoint cluster region (Bcr) is an important mediator of Ang II/PDGF induced VSMC proliferation [5]. We demonstrated that Bcr acts in part via

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Disclosures

None

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inhibition of peroxisome-proliferator-activated receptor gamma (PPAR γ) transcriptional activity. We have recently reported that Bcr binds to the RNA helicase, UAP56, and shown that interaction of Bcr with UAP56 is critical for Bcr induced VSMC DNA synthesis [6]. UAP56 is an ATP dependent RNA helicase with ATPase activity that is a member of the DExD/H box family of RNA helicases [7]. Like other DExD/H box proteins, UAP56 plays an important role in several steps of RNA synthesis and function including RNA splicing and mRNA transport from the nucleus to the cytoplasm [8–10]. Yamazaki et al. have shown that UAP56 forms an mRNA export machinery that regulates mitotic progression [11]. Knockdown of UAP56 results in down regulation of genes involved in the cell cycle, cell division, DNA repair and mitosis including the cell cycle regulator cyclin dependent kinase 2 (CDK2) [11]. Consistent with these findings, we found that while overexpression of Bcr increased the expression of the positive cell cycle regulator cyclin E and decreased the expression of the negative cell cycle regulator p21, a cyclin dependent kinase inhibitor, knockdown of UAP56 reversed this effect of Bcr on p21 and cyclin E expression [6].

While we have shown that Bcr is a major mediator of Ang II/PDGF induced VSMC proliferation, the role of UAP56 in Ang II/PDGF signaling is unknown. In the present study, we demonstrate that knockdown of UAP56 inhibits Ang II/PDGF induced DNA synthesis and VSMC proliferation. We have also observed that knockdown of UAP56 inhibits the transcriptional activation of the cell cycle regulator E2F and demonstrate that UAP56 is present in the vessel wall of low flow carotid arteries. These findings suggest that UAP56 is an important mediator of Ang II/PDGF signaling and may be a target for the treatment of vascular proliferative disease.

Materials and Methods

Cell culture

Rat VSMC were isolated as previously described [5, 12] or were purchased from Cell Applications, Inc. VSMC were maintained in DMEM containing 10% fetal bovine serum. Cells were treated with PDGF (R&D Systems) and Ang II (MP Biomedicals) as described in individual experiments.

siRNA transfection

For siRNA experiments, VSMC were transfected as previously described [13] with UAP56 siRNA oligonucleotides (Dharmacon-Smart pool) using Lipofectamine RNAiMAX (Invitrogen).

[³H] Thymidine Incorporation assay and cell counting

Measurement of [³H] thymidine incorporation into DNA was performed as previously described [5, 14]. Cell proliferation was quantitated by cell counting using a hemocytometer.

Western blotting

After treatment, the cells were washed with PBS and harvested in modified RIPA buffer containing protease inhibitor cocktail (Sigma). 50 μ g total protein lysates were separated on a 10% SDS-PAGE, transferred to a nitrocellulose membrane and immuno-blotted with UAP56 antibody (Santa Cruz) or tubulin antibody (Sigma) followed by horseradish peroxidase-conjugated secondary antibody (Amersham Life Science).

E2F transcriptional activity

E2F transcriptional activity was measured using a Cignal E2F Reporter (luc) kit (SA Biosciences). The cells were first transfected with control or UAP56 siRNA and 24 hours

later transfected with an E2F reporter. After serum starvation for 24 hours, cells were treated with PDGF (20 ng/ml). After 20 hours, cells were harvested and a dual luciferase assay was performed.

Carotid ligation and immunohistochemistry

Mice were used in accordance with the guidelines of the National Institutes of Health and the American Heart Association for the care and use of laboratory animals. All procedures were approved by the University of Rochester Animal Care Committee. Carotid ligation was performed as previously described [5, 15]. Briefly, mice of the Black Swiss background were anesthetized with an intraperitoneal injection of ketamine (130 mg/kg) and xylazine (8.8 mg/kg) in saline (10 mL/kg). The left external and internal carotid branches were ligated so that left carotid blood flow was reduced to flow via the occipital artery. Carotid arteries were harvested two weeks after ligation. For immunohistochemical analysis representative sections were stained for UAP56 (Lifespan Biosciences; 1:600 dilution). Analysis of relative expression of UAP56 (brown staining) was performed using ImagePro software version 6.2.

Statistics

Numeric data are expressed as means $\pm$ SD. Statistical analysis was performed with the StatView 5.0 package (ABACUS Concepts, Berkeley, Calif). Differences were analyzed with a 1-way or a 2-way repeated-measures ANOVA as appropriate, followed by Scheffé's correction for multiple comparisons. A probability value of <0.05 was considered significant.

Results

Knockdown of UAP56 inhibits Ang II/PDGF induced DNA synthesis and VSMC proliferation

We previously demonstrated that UAP56 is important in Bcr mediated DNA synthesis [6]. As Bcr is an important mediator of PDGF and Ang II signaling [5], we examined the role of UAP56 in Ang II/PDGF induced DNA synthesis. VSMC were transfected with UAP56 siRNA or control siRNA and then treated with Ang II (200 nM) and PDGF (20 ng/ml) for 6 hours. Knockdown of UAP56 expression significantly blocked Ang II/PDGF induced DNA synthesis assessed by [3 H] thymidine incorporation (Figure 1A). Evidence of knockdown of UAP56 expression with UAP56 siRNA in VSMC is shown in Figure 1B. Whereas PDGF is a potent stimulator of DNA synthesis, we used both Ang II and PDGF to stimulate the cells because we found that the combination of Ang II and PDGF maximized DNA synthesis [5]. To examine if this decrease in DNA synthesis correlated with a decrease in cellular proliferation, we used cell counting to examine the effect of UAP56 on VSMC proliferation. Knockdown of UAP56 with UAP56 siRNA inhibited Ang II/PDGF induced VSMC proliferation (Figure 2).

Knockdown of UAP56 inhibits E2F transcriptional activity

Yamazaki et al. have recently reported that knockdown of UAP56 down regulates several genes involved in cell cycle regulation including the cyclin dependent kinase CDK2 [11]. Similarly we have shown that knockdown of UAP56 affects the expression of cyclin E and the cyclin dependent kinase inhibitor p21 [6]. As cyclins and cyclin dependent kinases play an important role in the classical E2F pathway of cell cycle regulation, we next examined the effect of UAP56 on E2F activity. E2F transcription factors bind to DNA and regulate promoter expression [16, 17]. In the classical E2F pathway, retinoblastoma protein (Rb) binds to and inhibits E2F. When activated by mitogenic stimuli, cyclin/cyclin dependent kinase complexes phosphorylate Rb resulting in disassociation of Rb from E2F and

subsequent transactivation of genes involved in the cell cycle G1/S transition [16, 17]. Cyclin/CDK complexes are inhibited by cyclin dependent kinase inhibitors including p21 and p27. Using UAP56 siRNA we found that knockdown of UAP56 expression inhibited PDGF induced E2F transcriptional activity in VSMC (Figure 3). This suggests that UAP56 regulates DNA synthesis, at least in part, via E2F transcriptional activation.

UAP56 is expressed in the arterial wall after carotid ligation

Our findings suggest that UAP56 plays an important role in VSMC proliferation in vitro. We next examined UAP56 in a mouse carotid ligation in vivo model of intimal proliferation. The left external and internal carotid branches were ligated so that left carotid blood flow was reduced to flow via the occipital artery [5]. Ligated left carotid arteries and the non-ligated right carotid arteries were harvested two weeks after ligation. Carotid ligation was associated with development of intimal proliferation. No proliferation was seen in the non-ligated right carotid artery (Figure 4). We assessed UAP56 expression in carotid arteries in 4 mice following carotid artery ligation. Immunohistochemical analysis of left carotid artery ligation sections revealed positive staining for UAP56 in all animals (Figure 4). This staining was predominantly in the neointimal layer and was present in the nucleus as well as the cytoplasm. Using Image Pro Plus software we found that $31.3 \pm 3\%$ of the neointima stained positive for UAP56 in these animals. There was no neointima formation in the non-ligated right carotid artery and thus no neointimal staining of UAP56 in the right carotid artery. This demonstrates that UAP56 is expressed in proliferative lesions and marks UAP56 as a potential target for treating vascular proliferative diseases.

Discussion

The major findings of this paper are that the DExD/H box protein UAP56 is an important mediator of Ang II/PDGF induced DNA synthesis and VSMC proliferation and is also a positive regulator of PDGF-mediated E2F transcriptional activation. We have previously demonstrated that UAP56 is an important mediator of Bcr induced DNA synthesis. While Bcr is a mediator of VSMC proliferation in a mouse carotid ligation model, the role of Bcr in human vascular disease remains to be determined. Ang II and PDGF however, are known to play important roles in human vascular disease and our results define UAP56 as a potential target in the treatment of proliferative vascular disease. These findings further define the role of DExD/H box proteins in cell growth and proliferation. DExD/H box proteins regulate several stages of RNA metabolism including RNA export and translation [18, 19], regulate growth, and play important roles in transcriptional regulation. Several DExD/H box proteins are over expressed in cancer cells [18–21] and phosphorylation of the DExD/H box protein p68 is seen in several cancer cells but not the corresponding normal tissues [21]. p68 also plays an important role in PDGF induced cell proliferation by up-regulating cyclin D1 and c-Myc expression [22]. We have now demonstrated that UAP56 is another DExD/H box protein that is important in PDGF induced cell proliferation.

UAP56 is important for several steps of RNA metabolism including spliceosome assembly, mRNA export and protein synthesis [8–10, 13]. UAP56 plays an important role in cell growth and proliferation and our findings now demonstrate that UAP56 regulates E2F transcriptional activation and DNA synthesis in VSMC. UAP56 has also recently been reported to regulate mitosis [11]. Specifically, depletion of UAP56 was found to cause mitotic delay and sister chromatid cohesion defects. These findings along with this report demonstrate that UAP56 regulates several stages of the cell cycle.

DExD/H box proteins are thought to regulate cellular proliferation, in part via control of transcriptional regulation [18]. Our results suggest that UAP56 regulates cellular proliferation, at least in part via its effect on E2F transcriptional activation. E2F is a family

of transcription factors known to regulate cell cycle progression [23]. E2F plays a critical role in cell proliferation and inhibition of E2F blocks neointimal formation [24–26]. Several targets of E2F play important roles in cell cycle regulation and several of these targets including cyclin A and CDK2 are also targets of UAP56 [11, 27]. In addition to cell cycle regulation, E2F target genes are also regulators of DNA replication, mitosis and DNA repair, components of cellular growth that are also regulated by UAP56. There appears to be overlap between the functions of E2F and UAP56 and our finding suggest that UAP56 is a key component of E2F transcriptional activation.

Using a carotid ligation method, we have demonstrated presence of UAP56 in the vessel wall of low-flow carotid arteries. While UAP56 was predominantly present in the neointima, this does not preclude the possible importance of UAP56 in the medial layer or in endothelial cells. This finding also does not preclude the presence of UAP56 in the uninjured arterial wall. In addition to our in vitro findings, our findings do suggest that UAP56 may be important in the development of proliferative lesions. Further studies are needed to confirm this.

In conclusion, we demonstrate here that UAP56 is an important regulator of VSMC DNA synthesis and proliferation. UAP56 affects several aspects of cellular proliferation including DNA synthesis and progression of mitosis. This suggests that UAP56 may be an important target for treating proliferative diseases including vascular injury.

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Research Highlights

- Knockdown of UAP56 inhibits Angiotensin II/PDGF induced vascular smooth muscle cell proliferation.
- UAP56 is a positive regulator of E2F transcriptional activation.
- UAP56 is present in the vessel wall of low flow carotid arteries.

Figure 1. Knockdown of UAP56 inhibits Ang II/PDGF induced DNA synthesis in VSMC
 (A) Following transfection with control siRNA or UAP56 siRNA, rat VSMC were treated with Ang II (200 nM) and PDGF (20 ng/ml) or control for 6 hours. During the last hour of incubation the cells were pulse labeled with [³H] thymidine. Cells were then harvested and [³H] thymidine incorporation was measured. (**p<0.01 compared with control siRNA +PDGF + Ang II). The data are representative of triplicates using 3 different preparations of VSMCs. (B) Western blot demonstrating that UAP56 is present in VSMC and knockdown of UAP56 expression with UAP56 siRNA. Rat VSMC were treated with control or UAP56 siRNA for 72 hours. Western blot was then performed on whole-cell lysates with UAP56 antibody.

Figure 2. Knockdown of UAP56 inhibits Ang II/PDGF induced DNA VSMC proliferation
Following transfection with control siRNA or UAP56 siRNA, VSMC were serum starved for 48 hours and then treated with Ang II (200 nM) and PDGF (20 ng/ml) or control for 24 hours. Cells were then counted. (**p<0.01 compared with control siRNA +PDGF + Ang II). The data are representative of triplicates using 3 different preparations of VSMCs.

Figure 3. Knockdown of UAP56 blocks E2F transcriptional activation

VSMC were first transfected with control or UAP56 siRNA and 24 hrs later transfected with an E2F reporter. After serum starvation for 24 hrs, cells were treated with PDGF (20 ng/ml). After 20 hours, cells were harvested and a dual luciferase assay was performed. (** $p < 0.01$). The data are representative of triplicates using 2 different preparations of VSMCs.

Figure 4. Presence of UAP56 in the arterial wall after carotid ligation

Immunohistochemical analysis of left carotid artery ligation sections reveals staining for UAP56 in the intima of a carotid artery 2 weeks after ligation (red/brown stain) [Magnification 20x (A), Magnification 60x (B)]. Control IgG staining is shown in (C), Magnification 60x. Staining for UAP56 in the non-ligated right carotid artery is shown in (D), Magnification 60x. Arrows delineate the intima. (E) Quantification of relative UAP56 staining per area in the neointima and media in control (non-ligated right carotid artery) and ligated left carotid artery (n=4 animals).