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A repressor protein, Mnt, is a novel negative regulator of vascular smooth muscle cell hypertrophy by angiotensin II and neointimal hyperplasia by arterial injury

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Abstract

Objective—The Max-interacting protein Mnt is a transcriptional repressor that can antagonize the transcriptional and proliferation-related activities of Myc. Here, we tested the hypothesis that Mnt is a negative regulator of pathological vascular remodeling.

Methods—Adenovirus encoding Mnt or control GFP was infected to cultured rat vascular smooth muscle cells (VSMC) and carotid arteries after a balloon angioplasty.

Results—In VSMC, adenoviral gene transfer of Mnt suppressed angiotensin II-induced protein expression of early growth response protein-1 (Egr1) and its promoter activation. Mnt adenovirus did not interfere with upstream signaling of angiotensin II. Angiotensin II-induced protein accumulation in VSMC was inhibited by Mnt adenovirus. Mnt adenovirus also inhibited platelet-derived growth factor-induced VSMC proliferation. Moreover, Mnt adenovirus prevented neointima formation in response to arterial injury. The adenoviral Mnt gene transfer also prevented Egr1 induction in neointima.

Conclusion—These data identify Mnt as a previously unrecognized negative regulator of pathological vascular remodeling.

Keywords

transcriptional repressor; angioplasty; gene therapy; restenosis; signal transduction

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Author contribution

Takehiko Takayanagi, Akito Eguchi, Akira Takaguri, and Allison M. Bourne researched the data and contributed to the discussion. Peter J. Hurlin provided reagents and Peter J. Hurlin and Katherine J Elliott edited the paper and contributed to the discussion and revision. Satoru Eguchi designed the study and wrote the paper.

1. Introduction

The Mxd-related nuclear transcriptional repressor protein, Mnt, is expressed ubiquitously in mammalian organs and Mnt orthologs have conserved structure and repressor functions from drosophila to human. Mnt heterodimerizes with Max through basic helix-loop-helix leucine zipper domains and can compete with Myc for binding to Max [1]. In biological assays, ectopic Mnt expression can antagonize cell transformation by Myc [2]. Gene deletion and knockdown studies show that Mnt is both a negative regulator of proliferation and has prosurvival activity. Both of these activities may derive from its ability to antagonize Myc [1]. Studies in both mice and drosophila reveal that Mnt governs cell growth and organ size [1], but it is currently unknown whether Mnt has any regulatory role in cardiovascular physiology and pathophysiology.

Of the key genes/proteins associated with pathological vascular remodeling, the essential role of the early growth response-1 (Egr1) in growth and migration of vascular smooth muscle cells (VSMC) has been demonstrated *in vitro* in cell culture as well as in neointima formation in response to vascular injury *in vivo* [3]. Also, Egr1 has been shown to control growth and migration regulatory genes such as those of platelet-derived growth factor (PDGF) and tissue growth factor β in VSMC [3]. In addition, Egr1 is rapidly induced by angiotensin II (Ang II) stimulation in VSMC [4]. Interestingly, a recent promoter array approach suggests that Mnt binds to and regulates the *Egr1* promoter [5]. In the present study, we have tested our hypothesis that Mnt is a negative regulator of pathological vascular remodeling by preventing Egr1 induction.

We found that Mnt is expressed in VSMCs and additional gene transfer of Mnt inhibited growth promoting effects of Ang II and PDGF-BB. Mnt gene transfer also inhibited neointimal hyperplasia of carotid artery in response to balloon angioplasty. These Mnt responses are associated with suppression of Egr1 induction. These data suggest a novel therapeutic potential of Mnt gene transfer to prevent vascular remodeling likely via its Egr1 repression.

2. Methods

For detailed methodology, please refer to the data supplement.

2.1. Adenoviral infection of VSMCs

Replication-deficient adenovirus encoding C-terminal HA tagged mouse Mnt [2] was created by using pAd/CMV/V5-DEST™ Gateway Vector Kit as described previously [6]. Rat aortic VSMCs at passage 3–10 at 80~90% confluence in culture wells were made quiescent by incubation with serum-free medium for 24 h before the adenovirus infection. VSMC were infected with adenovirus for 2 days as previously described [6] with a modification to include 3% FuGENE 6 for the infection medium. The infection efficiency was estimated to be 90–100% as defined by infection with adenovirus (100 moi) encoding green fluorescent protein (GFP).

2.2. Balloon angioplasty and adenoviral gene transfer

Left common carotid artery balloon angioplasty was performed in male Sprague-Dawley rats (Charles River Breeding Laboratory) as previously reported [7]. After balloon injury, a cannula was introduced into the common carotid artery and the distal injured arterial segment isolated by temporary clips placed midway in the injured segment and at the orifice of the internal carotid artery. This space was filled with 100 μL of the adenovirus encoding Mnt or control GFP (2×10^9 pfu/mL). Incubation was allowed to proceed for 15 min and then the solution was retrieved, the cannula removed, blood circulation restored, and the wound

closed. The vessels were harvested 14 days post-delivery, fixed, and histology was determined as described [7]. Protein expression of adenoviral-encoded GFP in the transferred arteries was confirmed 14 days after the delivery (Figure S1). These investigations conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and Temple University [7].

3. Results

Primary and quiescent cultured rat VSMC express Mnt, and its protein expression levels remain unchanged up to 24 h after 100 nM Ang II stimulation (Figure S2). To study the potential repressor role of Mnt in growth promoting gene induction in VSMC, adenoviral vector encoding Mnt was created. In VSMC, adenoviral gene transfer of Mnt (100 moi) markedly suppressed Ang II-induced protein expression of Egr1 (Figure 1A) and c-Fos (Figure S3). Neither adenovirus nor Ang II affected c-Myc expression. Mnt adenovirus also suppressed Ang II-induced promoter activation of Egr1 (Figure 1B). However, Mnt adenovirus did not interfere with upstream signal transduction of Ang II in VSMCs such as epidermal growth factor (EGF) receptor transactivation or extracellular signal-regulated kinase (ERK) 1/2 phosphorylation (Figure 1C).

To study the regulatory roles of Mnt in VSMC hypertrophy and proliferation, VSMCs were stimulated with Ang II or PDGF, respectively. Ang II-induced protein accumulation in VSMC was inhibited by Mnt adenovirus compared with control GFP adenovirus (Figure 2A). Neither, the adenovirus nor Ang II affected cell number in these conditions (Figure S4). Mnt adenovirus also inhibited PDGF- or 2.5% fetal calf serum-induced VSMC proliferation (Figure 2B, S5 and S6). To study the role of endogenously expressed Mnt in vascular biology, adenoviral vectors encoding artificial microRNAs targeting rat *Mnt* were created. While this approach significantly silenced Mnt in VSMC without affecting expression of GAPDH, Mnt silencing was associated with significant reduction in c-Myc expression in the cells (Figure S7).

Mnt was faintly stained in control carotid artery, whereas significant nuclear Mnt staining was observed in neointima of the injured carotid artery (Figure S8). To test whether Mnt prevents pathophysiological vascular remodeling, the carotid artery neointima formation upon arterial injury was evaluated with Mnt or control adenovirus gene transfer. As shown in Figure 2C, Mnt adenovirus markedly prevented neointima formation in response to arterial balloon injury *in vivo* (intima/media ratio, GFP 1.18±0.28 vs Mnt 0.26±0.02, $p<0.05$). Egr1 induction was observed in neointima after control GFP adenovirus gene transfer, but Egr1 was not observed in sections with Mnt adonvirus gene transfer (Figure 2D).

4. Discussion

Results of *in vitro* and *in vivo* studies presented here suggest that Egr1 is a Mnt-regulated gene in VSMCs. Ang II-activated ERK1/2 increases Egr1 transcription through serum response elements located in the promoter [8]. While the exact binding site has not yet been identified, Mnt likely binds to Egr1 promoter and represses its transcription. Egr1 inhibition or silencing has been reported to diminish intimal hyperplasia after arterial injury and cardiac hypertrophy in response to pressure overload [3]. However, we observed that Ang II-induced c-Fos induction was also reduced by Mnt overexpression. The effects of Mnt on Ang II-induced oxidative stress and inflammatory response, both believed to be critical for target organ damage [9], remain unclear. Therefore, the suppression of Egr1 induction is currently one of the potential mechanisms through which Mnt could act to reduce pathological vascular remodeling.

We are aware that the role of endogenously expressed Mnt in vascular biology is a critical issue to be studied further. Mnt acts as an endogenous Myc antagonist [1]. In human endothelial cells, Mnt was identified among the genes induced by c-Myc suggesting a feedback relationship between c-Myc and Mnt [10]. This correlates with Mnt induction in the neointima observed in the present study since Myc induction in the neointima has been reported [11]. We also observed that Mnt reduction lead to significantly less expression of Myc in VSMC. The reduction in c-Myc is consistent with previous studies showing that c-Myc is markedly reduced in Mnt deficient whole embryos [12] and embryonic fibroblasts [13]. It is likely that the cells have a negative feedback mechanism to maintain Myc expression below basal when Mnt levels become abnormally low. However, this relationship was not observed in case of Mnt overexpression. Myc was reported to regulate Egr1 transcription [14]. Myc inhibition also prevented intimal hyperplasia and pathophysiological cardiac hypertrophy [11]. Therefore, the mechanism underlying these pathophysiological responses may involve positive and negative regulation of Egr1 through Myc and Mnt, respectively, together with the still to be elucidated feedback mechanism between Myc and Mnt [15].

In conclusion, we show that Mnt has the potential to function as a negative regulator of pathological vascular remodeling. Increasing Mnt expression or its downstream effectors may have therapeutic potential toward preventing cardiovascular diseases, at least in part, by suppressing Egr1 induction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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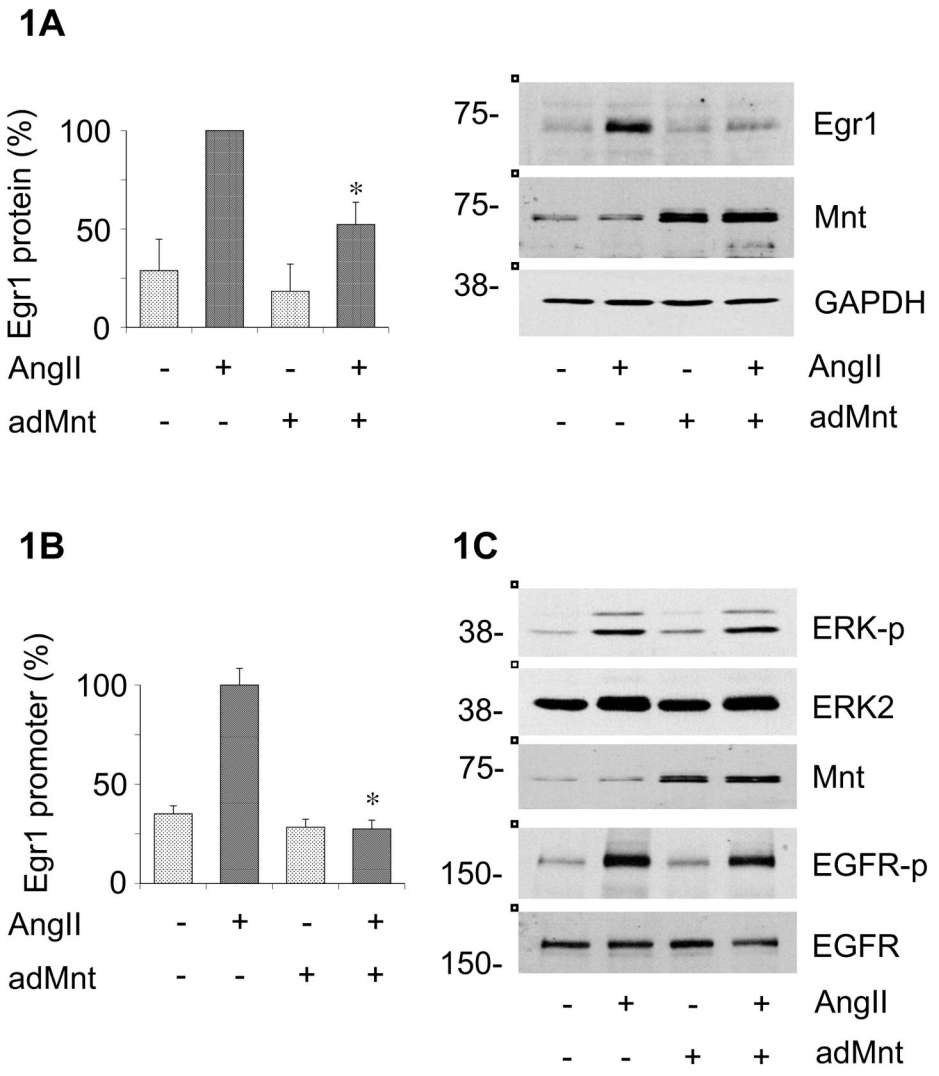

Fig. 1.

Effects of Mnt gene transfer by adenovirus on Ang II signals in VSMC. VSMC were infected with adenovirus expressing Mnt or control GFP at 100 moi for 2 days. **A**, The cells were stimulated with 100 nM Ang II for 1 hour. Cell lysates were analyzed by immunoblotting with antibodies as indicated. The densitometry analysis of the data is from quadruplicated experiments (mean ± SEM, * $p < 0.05$ compared with stimulated control). Representative blots are shown. **B**, After the adenoviral infection, cells were cotransfected with the *egr-1* promoter luciferase construct and control vector (SEAP). Serum-deprived cells were incubated with 100 nM Ang II for 24 hours. Bars represent % induction in normalized luciferase activity from quadruplicated experiments (mean ± SEM, * $p < 0.05$ compared with stimulated control). **C**, The cells were stimulated with 100 nM Ang II for 10 min (ERK-p, ERK2 and Mnt) or 2 min (EGFR-p and EGFR). Cell lysates were analyzed by immunoblotting with antibodies as indicated. Representative blots are shown from the quadruplicated experiments.

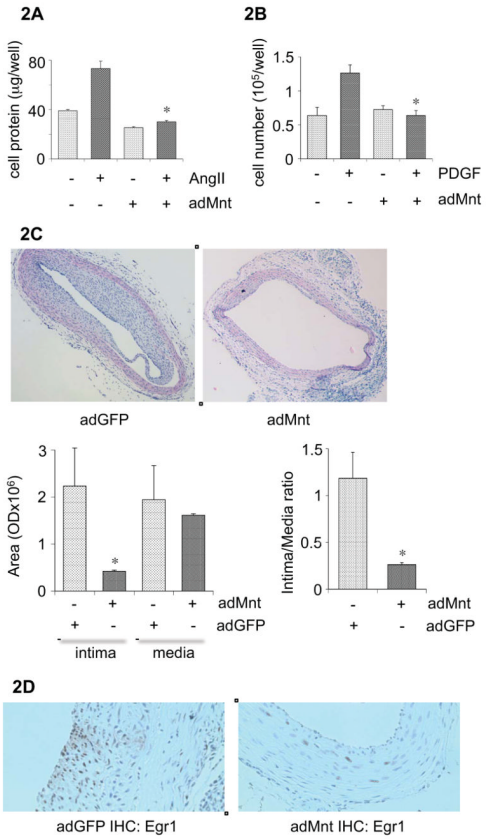

Fig. 2.

Mnt gene transfer inhibited hypertrophy and proliferation of VSMC *in vitro* and neointimal hyperplasia *in vivo*. **A,** Subconfluent VSMC infected with adenovirus (100 moi) encoding Mnt or the control GFP vector were stimulated with Ang II for 3 days and cellular protein contents were determined as a marker of hypertrophy from quadruplicated experiments (mean ± SEM, * p <0.05 compared with stimulated control). **B**, VSMC were infected with adenovirus (100 moi) encoding Mnt or control GFP vector for 2 days. The cells were then stimulated with 25 ng/mL PDGF-BB for 3 days and cell numbers were determined by a Coulter counter from quadruplicated experiments. Data are mean±SEM. *$p < 0.05$ compared to the stimulated control. **C,** Mnt adenovirus inhibited arterial neointima formation after balloon injury. Representative sections (x40 magnification) are shown. 14 days after the injury, common carotid artery was stained and the area of neointima and media were quantified. Data are mean ± SEM of sections from 4 rats. *p<0.05 compared to the GFP adenovirus-infected control. **D**, Arterial sections obtained on day 14 after injury were stained with Egr1 antibody (x200 magnification). Representative sections (x200 magnification) are shown.