Original Article

Role of the Angiotensin II Type 2 Receptor in Arterial Remodeling after Wire Injury in Mice

Yoshiyuki YAMAMOTO[1)], Yuichiro WATARI[1)], Andrei BRYDUN[1)], Masao YOSHIZUMI[2)], Masahiro AKISHITA[3)], Masatsugu HORIUCHI[4)], Kazuaki CHAYAMA[1)], Tetsuya OSHIMA[5)], and Ryoji OZONO[5)]

The angiotensin II type 2 (AT2) receptor promotes apoptosis and inhibits cell proliferation. In the present study, we investigated the role of the AT2 receptor in vascular repair and remodeling following severe vascular injury using AT2 knockout (AT2KO) mice. Left femoral arteries of AT2KO mice and wild-type (WT) control mice were injured by a 0.38 mm steel wire inserted from the lumen. Twenty-eight days after the injury, a concentric vascular wall thickening, composed largely of neointima, was noted both in AT2KO and WT mice. The area occupied by the neointima and the cell count within it were not different in the two mouse strains. However, the area of the medial layer and the cell count within it were significantly larger in AT2KO mice than in WT mice. A BrdU incorporation assay showed that the proliferative activity was high in the neointima but it was not different between the strains. On the other hand, apoptosis assessed by terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling (TUNEL) was significantly inhibited in the neointima and the media of AT2KO mice compared to the levels in WT mice. However, the number of TUNEL-positive cell was much smaller in the neointima than in the medial layer in both strains. Taken together, these results indicate that AT2 receptors promote the apoptosis of vascular cells but have no net effect on the neointimal cell growth or luminal narrowing after wire injury. The AT2 receptor may be involved in the control of medial layer thickness, at least in part, through medial cell apoptosis. (*Hypertens Res* 2008; 31: 1241–1249)

***Key Words*: angiotensin II type 2, receptor, restenosis, vascular smooth muscle**

Introduction

Balloon angioplasty and stent implantation are the most widely used techniques for treating coronary artery stenosis. However, mechanical injury to the vascular lumen occurs during angioplasty and causes neointimal hyperplasia and vascular remodeling, resulting in restenosis of the culprit lesion. Although the introduction of drug-eluting stents has contributed to a reduction in the rate of restenosis, these stents cannot be used for some patients (*1*). The mechanism of restenosis has still not been fully elucidated.

Angiotensin II has two major receptor subtypes, the angiotensin II type 1 (AT1) receptor and angiotensin II type 2 (AT2) receptor. It has been reported that specific blockade of the AT1 receptor or deletion of the AT1 receptor gene in mice caused amelioration of restenosis after vascular injury (*2–4*). On the other hand, the AT2 receptor is thought to counteract

From the [1)]Departments of Medicine and Molecular Science, [2)]Cardiovascular Physiology and Medicine, and [5)]Clinical Laboratory Medicine, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Japan; [3)]Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan; and [4)]Department of Biochemistry, Ehime University School of Medicine, Toon, Japan.

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (17590493) and grants from the Kurozumi Medical Foundation and Takeda Science Foundation.

Address for Reprints: Ryoji Ozono, M.D., Ph.D., Department of Clinical Laboratory Medicine, Hiroshima University Graduate School of Biomedical Sciences, 1–2–3 Kasumi, Minami-ku, Hiroshima 734–8551, Japan. E-mail: ozono@hiroshima-u.ac.jp

Received August 28, 2007; Accepted in revised form February 14, 2008.

the signals transmitted by the AT1 receptor, eliciting vasodilatation (*5*), inhibition of proliferation (*6–8*), NO production (*9*) and apoptosis (*10–14*). Therefore, it has been hypothesized that specific stimulation of the AT2 receptor may inhibit the process of restenosis after vascular injury. Recently, specific AT1 receptor blockers (ARBs) have been widely used to treat hypertension and heart failure. Specific blockade of the AT1 receptor with an ARB results in elevation of circulating angiotensin II and thus an overstimulation of the AT2 receptor (*15*), indicating that the effect of an ARB might be partly mediated by its effect on the AT2 receptor (*16*). However, the roles of the AT2 receptor in the repair and remodeling of an injured artery are not well understood. There have been conflicting observations (*4*, *10*) regarding the benefits of AT2 receptor stimulation after vascular injury.

In this study, we investigated the role of the AT2 receptor in the process of stenotic lesion formation following severe vascular injury that mimics that caused by clinical balloon angioplasty, focusing on the proliferation and apoptosis of vascular smooth muscle cells.

Methods

Animals and Study Protocol

Adult male wild-type (WT) mice and AT2 knockout (AT2KO) mice, 10 to 14 weeks of age, were used in this study. After measuring body weight, blood pressure, and heart rates, mice were anesthetized with pentobarbital and underwent surgery to create an injury in the femoral artery with a steel wire. Blood pressure and heart rates were measured by the tail-cuff method as previously described (*9*, *17*). Mice were killed by overdose administration of pentobarbital 2 h, 3 d, 14 d and 28 d after the wire injury to obtain the femoral arteries.

The AT2KO mice were generated by Hein *et al*. (*18*) and kept in the Department of Biochemistry, Ehime University School of Medicine as previously described (*10*). The animals were sent to Hiroshima University, where all the experiments were conducted. The AT2KO mice were back-crossed for at least 6 generations onto a C57 background. All experimental procedures were approved and carried out in accordance with the Guidelines of Hiroshima University Graduate School of Biomedical Sciences.

Wire Injury Model

This model was developed by Sata *et al*. (*19*) and is described in detail in an on-line video (http://plaza.umin.ac.jp/~msata/). Briefly, the left femoral artery was exposed by blunted dissection. The femoral artery and vein were looped together proximally and distally with a 7-0 silk suture (Natsume Co., Tokyo, Japan) for temporary vascular control during the procedure. A small branch between the rectus femoris and vastus medialis muscles was isolated and ligated distally with a 7-0 silk suture. Veins and connective tissues around the artery were carefully removed. Transverse arteriotomy was performed in the muscular branch with Vannas style iris spring scissors (Fine Science Tools, Inc., Foster City, USA). Microsurgery forceps (Natsume) were used to extend the arteriotomy, and a straight spring wire (0.38 mm in diameter, No. C-SF-15-15; COOK, Bloomington, USA) was carefully inserted into the femoral artery for a distance of more than 5 mm toward the iliac artery. The wire was left in place for 1 min to denude and dilate the artery. Then the wire was removed and a silk suture looped at the proximal portion of the muscular branch artery was secured. Blood flow in the femoral artery was restored by releasing the sutures placed in the proximal and distal femoral portions. The skin incision was closed with a 5-0 silk suture.

Histological and Morphometrical Analysis

The injured and uninjured femoral arteries and surrounding tissues were excised after perfusion *via* the left ventricle with 0.9% NaCl solution followed by 4% paraformaldehyde in phosphate-buffered saline, then post-fixed in 4% paraformaldehyde overnight at 4°C. Tissues were cut in the middle of the femoral arteries, embedded in paraffin, and sectioned at 4 μm in thickness. Specimens taken from animals maintained for 28 d after injury were stained with hematoxylin and eosin (HE) and by the Elastica van Gieson staining protocol. The total areas of the intima, media, and lumen within a cross-section of the artery in the slides stained with Elastica van Gieson were calculated using software (Flovel Filing System; Olympus, Tokyo, Japan). For analysis of the net effect of cell proliferation and death, we counted the cell number in each component of the intima and media in the slides stained with HE. We manually counted all of the intimal or medial cells within a cross-section of the artery using a counter included in the Flovel Filing System software. The count from the entire cross-section was regarded as a single value. The area and cell number were evaluated using at least 2 slides per artery and the mean values were used for statistical analysis.

Immunohistochemistry

Sections were immunohistochemically stained for α-actin (monoclonal mouse anti–α smooth muscle actin antibody, at a dilution rate of 1:800; Sigma, St. Louis, USA) as a marker for vascular smooth muscle cells, for platelet–endothelial cell adhesion molecule-1 (PECAM-1; monoclonal rat anti-mouse CD31/PECAM-1 antibody, at a dilution rate of 1:200; Pharmingen, San Diego, USA) as a marker for endothelial cells, for Bcl-2 (polyclonal rabbit anti-mouse Bcl-2 antibody, at a dilution rate of 1:800; Pharmingen) and for the angiotensin II type 1 receptor (polyclonal rabbit anti-human angiotensin II type 1 receptor, at a dilution rate of 1:800; Assay Designs, Ann Arbor, USA). Sections were de-paraffinized, rinsed in xylene, and rehydrated. They were then blocked with 3% H_2O_2,

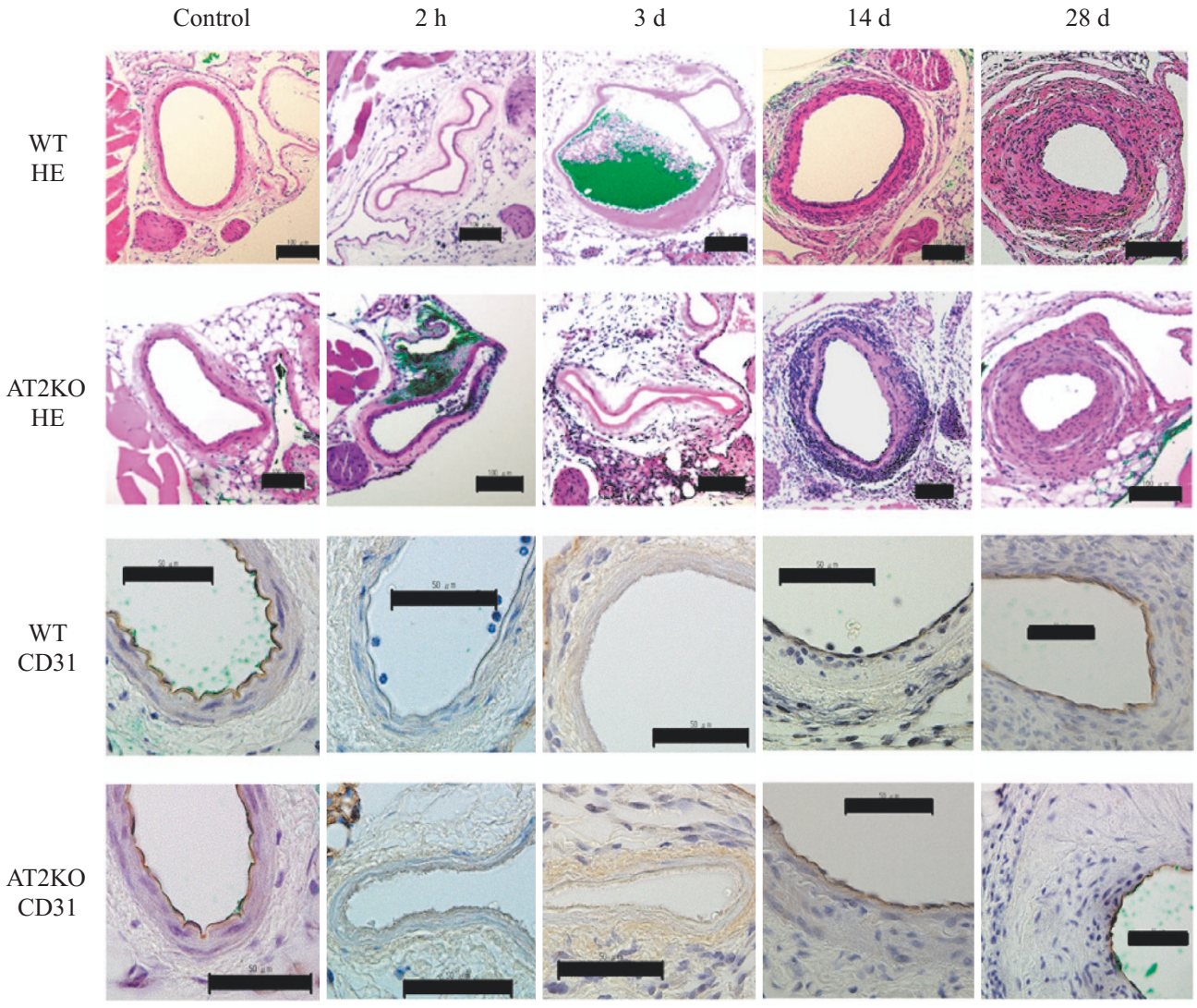

Fig. 1. *Femoral arteries of WT and AT2KO mice taken before (control) and after the wire injury (2 h and 3, 14 and 28 d after the injury). HE: hematoxylin and eosin staining. Magnification ×100. Bar = 100 μm. CD31: immunohistochemical staining of the endothelium with CD31. Magnification ×400. Bar = 50 μm.*

washed in PBS, and incubated with primary antibodies at 37°C for 2 h or at 4°C overnight. After being washed in PBS, sections were treated with a biotinylated anti-mouse or anti-rat or anti-rabbit IgG for 1 h at room temperature. Sections were then washed again in PBS, reacted with horseradish peroxidase–conjugated streptavidin, and developed with 3,3′-diaminobenzidine. After a final washing in distilled water, all sections were counterstained with hematoxylin.

In Vivo Bromodeoxyuridine Cell Proliferation Assay

Samples for the bromodeoxyuridine (BrdU) assay were taken 14 d after the wire injury. BrdU (Sigma) at a dose of 30 mg/kg was intraperitoneally injected twice, 48 h and 24 h prior to death. Immunohistochemistry using anti-BrdU antibody (BrdU Immunohistochemistry System; Calbiochem, La Jolla, USA) was performed according to the manufacturer's protocol. The sections were counterstained with hematoxylin. We counted the total numbers of BrdU-positive nuclei and the total number of nuclei in each component of the intima and media. The count from the entire cross-section of the artery was regarded as a single value. The BrdU index was defined as the ratio (%) of the number of BrdU-positive nuclei within the intima or media to the entire number of nuclei within the intima or media, respectively.

In Situ Detection of Apoptotic Cells

Apoptotic cell death was evaluated by terminal deoxynucle-

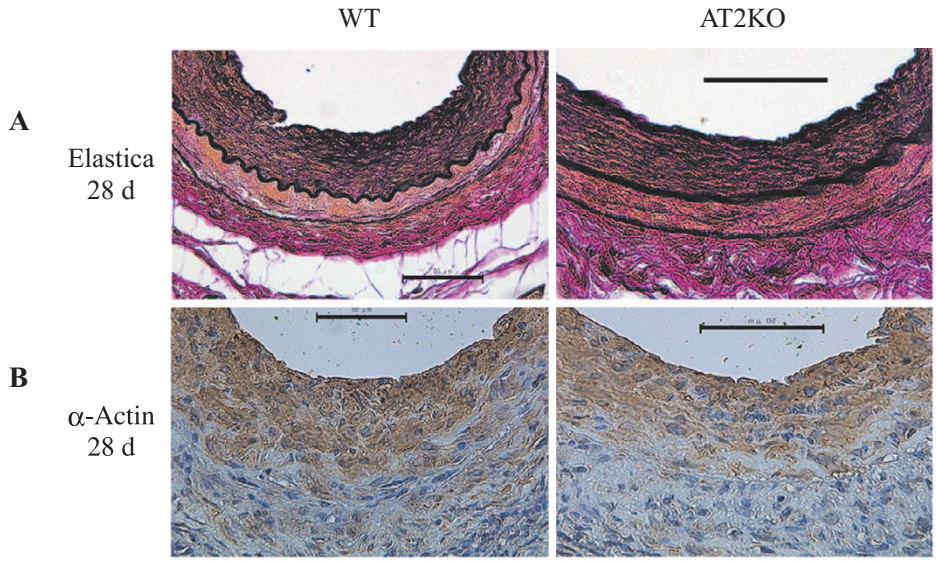

Fig. 2. *Femoral arteries of WT and AT2KO mice taken 28 d after wire injury. A: Elastica van Gieson staining (Elastica). B: α-Actin staining (α-Actin). Magnification ×400. Bar = 50 μm.*

otidyl transferase–mediated dUTP-biotin nick end labeling (TUNEL). Sections were stained using an *in situ* cell death detection kit (ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit; Intergen, Purchase, USA) according to the manufacturer's protocol. The sections were counterstained with methyl green. We counted the number of TUNEL-positive cells and the total number of the cells in each component of the intima and media in cross-sections of vessels. The count from the entire cross-section of the artery was regarded as a single value. The TUNEL index was defined as the ratio (%) of the number of TUNEL-positive cells within the intima or media to the total number of cells within the intima or media, respectively. The morphological changes of nuclear chromatin were examined by staining chromatin with Hoechst 33342 (5 μg/mL in PBS) for 30 min at 37°C and viewing the section under a fluorescence microscope.

Statistical Analysis

All results are expressed as means±SEM. Two investigators blinded to the genotype made all measurements and averaged the values. Differences were evaluated with Student's *t*-test and a value of $p<0.05$ was considered significant.

Results

Repair and Remodeling of the Arterial Wall after Wire Injury

There were no significant differences in body weight (WT: 26.2±0.4g; AT2KO: 26.8±0.5g), systolic and diastolic blood pressures (WT: 109.8±13/80.8±11 mmHg; AT2KO: 109.9±11/75.2±9 mmHg), or heart rate (WT: 509.6±65 bpm; AT2KO: 551.3±112 bpm) between WT and AT2KO mice. Figure 1 shows histology and immunohistochemical staining of the endothelium with CD31 in uninjured right arteries and injured left femoral arteries from WT and AT2KO mice. Femoral arteries of both WT and AT2KO mice excised 2 h after wire injury showed complete denudation of the endothelium, which was confirmed by the absence of CD31 staining. Three days after wire injury, the endothelium was still absent and marked thinning of the medial layer was observed in sections from both WT and AT2KO mice. Fourteen days after wire injury, re-endothelialization (CD31 staining) and neointimal formation were noted in most of the arterial sections, the frequency and extent of which were not apparently different between WT and AT2KO mice. In sections taken 28 d after injury, marked thickening of the arterial wall associated with narrowing of the luminal area was noted both in WT and AT2KO mice. At this point, re-endothelialization was completed in both strains.

Quantitative Analysis of Intimal and Medial Thickening after Wire Injury

To analyze the localization-specific roles of AT2 receptors within the vascular wall, the areas of vascular wall components were quantitatively analyzed in arteries of WT and ATKO mice taken 28 d after injury. In all of the vascular wall sections, internal and external elastic laminae were identifiable by Elastica van Gieson staining (Fig. 2A). Neointima and media mainly consisted of vascular cells positive for α-smooth muscle actin (Fig. 2B). A quantitative analysis revealed that the vascular injury caused marked thickening of

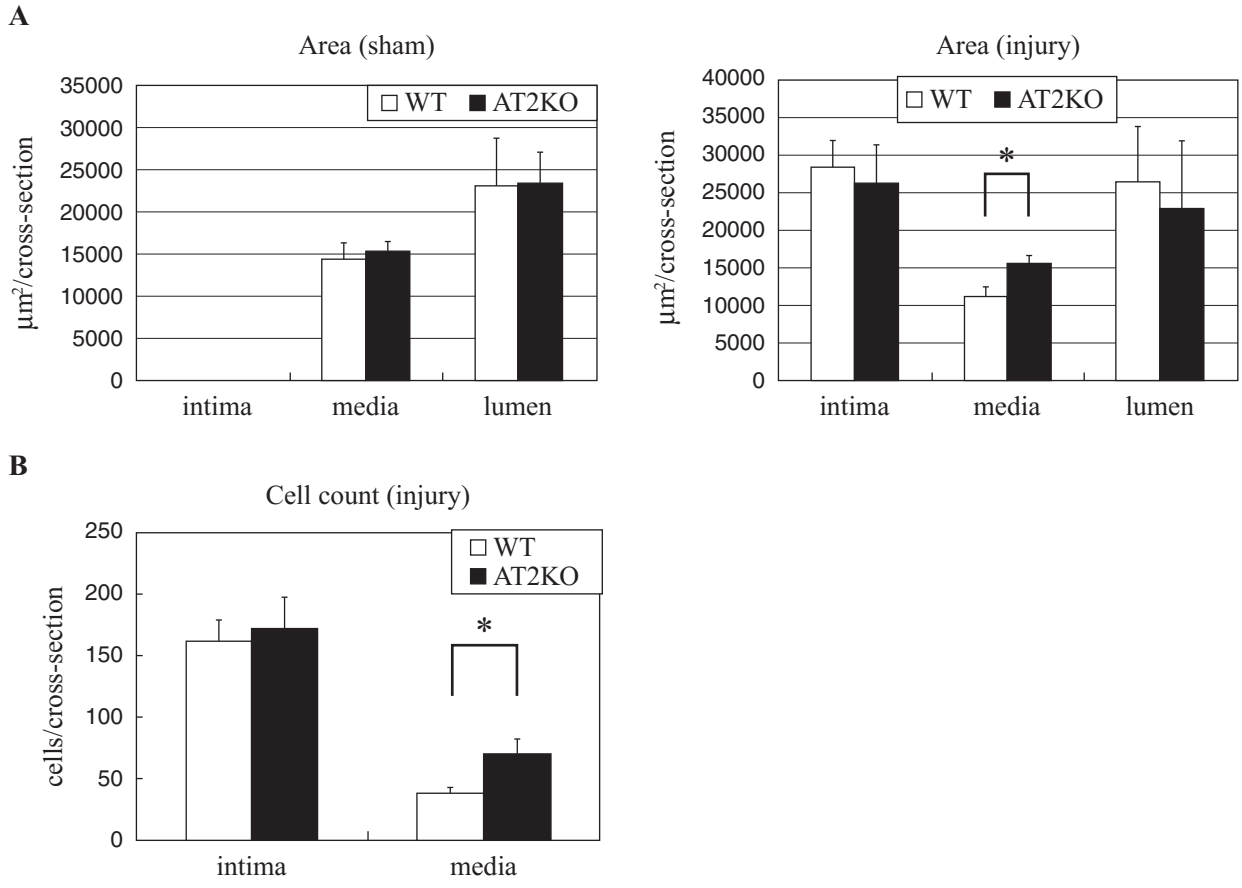

Fig. 3. *A: Bar graph showing areas of the intima, media and lumen of the femoral artery from WT (n = 14) and AT2KO mice (n = 10). The arteries were excised 28 d after sham operation (left) or wire injury (right). In arteries from sham-operated animals, the intima was not sufficiently thickened for the area to have a positive value. B: Bar graph showing vascular cell count within the intima and media of the injured femoral artery. The arteries were excised 28 d after wire injury. *p < 0.05. Each bar represents mean ± SEM.*

the intima both in WT and AT2KO mice, whereas the areas of the intima were not significantly different between the two strains (Fig. 3A). However, the medial area was significantly larger in AT2KO mice and the intima/media ratio was significantly smaller in AT2KO mice. As a result, the luminal area was slightly smaller in AT2KO mice, but the difference did not reach the level of statistical significance. Next, we counted the cell number in each component of the intima and media. Consistent with the results of analyses of intimal and medial thicknesses, the cell number in the media in AT2KO mice was significantly larger than that in WT mice (Fig. 3B), whereas the cell number in the intima was not different in the two strains. These results suggest that thickening of the media in AT2KO mice was not a result of accumulation of extracellular matrix and that the AT2 receptor is involved in the mechanism of the control of vascular cell proliferation or death following injury, at least in the media.

AT2 Receptor Did Not Influence Proliferation of Vascular Cells

To further investigate the mechanism of increase in medial cells in AT2KO mice, we performed a BrdU cell proliferation assay. Samples for the BrdU assay were taken 14 d after injury, when the neointima had started to thicken. In both of the strains, BrdU-positive cells were frequently observed in the neointima but rarely observed in the media, suggesting that active proliferation of vascular cells occurred only in the intima (Fig. 4A). However, the BrdU index in the neointima or media was not different between the two strains of mice (Fig. 4B), suggesting that the AT2 receptor is not involved in the mechanism of vascular cell proliferation after vascular injury, either in the neointima or media.

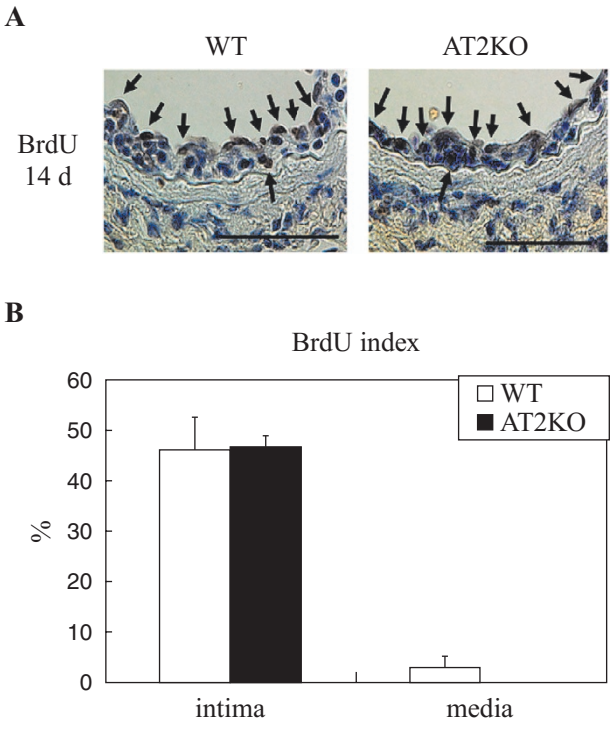

Fig. 4. *A: BrdU incorporation in the femoral arteries of WT and AT2KO mice. The arteries were excised 14 d after wire injury. Magnification ×400. Bar = 50 μm. The arrows indicate BrdU positive nuclei (brown). B: Bar graph showing the BrdU index (BrdU positive nuclei/total nuclei) in femoral arteries of WT (n = 6) and AT2KO mice (n = 4). The arteries were excised 14 d after wire injury. Each bar represents mean ± SEM.*

AT2 Receptor Increased TUNEL-Positive Cells after Wire Injury

Next, we investigated cell death in arterial sections taken at 2 h, 3 d, 14 d and 28 d after injury (Fig. 5A). The numbers of TUNEL-positive cells were analyzed separately in the intima and media (Fig. 5B). TUNEL assays in the intima were possible only at 14 and 28 d after injury, since the intima was absent before then. In both strains of mice, the number of TUNEL-positive cells in the intima was less than one-third of that in the media at any time point, suggesting that apoptosis was relatively inhibited, or some cell survival program was activated, in the intima (Fig. 5B). There was a common tendency in WT and AT2KO mice for the number of TUNEL-positive cells to decrease with time.

Focusing on the difference between WT and AT2KO mice (Fig. 5B), the TUNEL index was significantly smaller in AT2KO mice than in WT mice both in the intima and in the media in sections taken 2 h after injury, 3 d after injury and 14 d after injury.

As TUNEL assay may detect cell death other than apoptosis, we performed chromatin dye staining of injured arteries. Findings specific to apoptosis, including chromatin condensation and nuclear fragmentation into apoptotic bodies, were more frequently observed in WT mice (Fig. 5C). Furthermore, immunohistochemical staining for Bcl-2, an antiapoptotic protein, tended to be increased in the injured arteries of AT2KO mice (Fig. 5D).

These results are consistent with the notion that lack of the AT2 receptor causes attenuation of apoptosis in both the intima and media of the vascular wall.

Expression of the AT1 Receptor

To determine whether the alteration of cell proliferation/death in vascular cells of AT2KO mice resulted from upregulation of the AT1 receptor in the mutant mice, we studied how the AT1 receptor was expressed in the injured arteries of AT2KO and WT mice. The AT1 receptor was expressed both in the neointima and media throughout the experimental periods, but the expression levels did not differ between AT2KO and WT mice (Fig. 6), suggesting that a decrease in TUNEL-positive cells and an increase in the total cell count of the medial layer were unlikely to be mediated by the AT1 receptor.

Discussion

We demonstrated that TUNEL-positive cells were significantly decreased in AT2KO mice both in intimal and medial cells following severe vascular injury. This finding supports the notion that the AT2 receptor promotes apoptosis, thereby antagonizing the neointimal thickening and lumen narrowing (*10*). However, in the present study, neither the neointimal thickness nor the lumen size in AT2KO mice was significantly different from that in WT mice. In the neointima, irrespective of the mouse strain, the BrdU incorporation was much more active and the number of TUNEL-positive cells was much smaller than in the media, suggesting that the severe wire injury caused full activation of the proliferative program and inhibition of the cell death program, including apoptosis in intimal cells. Therefore, the AT2 receptor–mediated apoptosis, if any, does not appear to be the major determinant of neointimal thickening under our experimental conditions. The reason why the AT2 receptor had no effect on BrdU incorporation is unclear. However, AT2KO mice displayed a significant thickening of the medial layer which was associated with a reduced number of TUNEL-positive cells, suggesting that the stimulation of AT2 receptors may contribute to the prevention of luminal narrowing, at least in part, through inhibition of apoptosis.

The results of recent clinical trials (*2*, *3*) support the notion that AT2 receptor stimulation may contribute to the inhibition of restenosis following balloon angioplasty. In the VALPREST trial (*2*) and VALPACE trial (*3*), AT1 receptor blockade with ARBs, which have actions as AT2 receptor stimulators (*16*), prevented restenosis of the coronary lesion

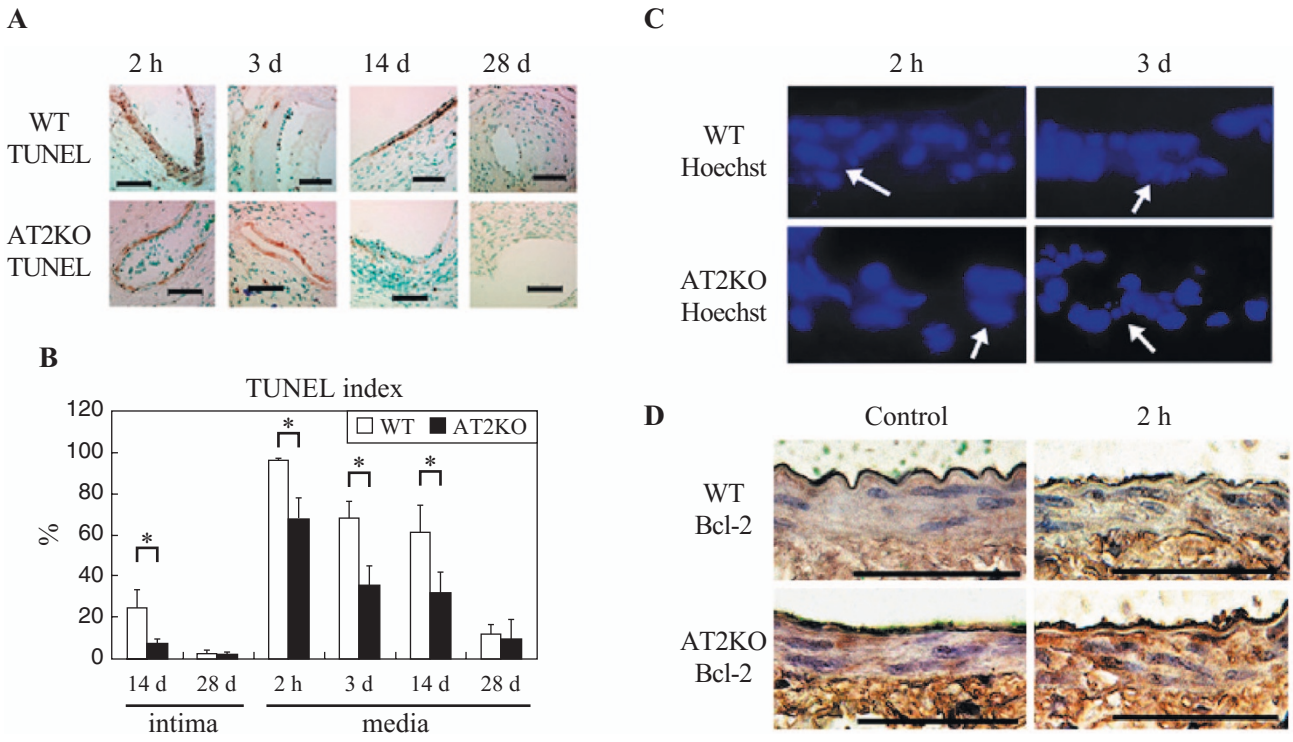

Fig. 5. *Apoptosis in femoral arteries of WT and AT2KO mice following wire injury. A: TUNEL staining. Sections were counterstained with methyl green. Magnification ×400. Bar = 50 μm. B: Bar graph showing the TUNEL index (TUNEL positive nuclei/total nuclei) in the intima and media of femoral arteries excised from WT (n = 5–6) and AT2KO mice (n = 5–6) at the indicated time points after wire injury. *p < 0.05. Each bar represents mean ± SEM. C: Femoral arteries of WT and AT2KO mice (2 h and 3 d after injury) stained with Hoechst 33342 (Hoechst). Magnification ×1,000. The arrows indicate chromatin condensation and fragmentation into apoptotic bodies. D: Immunohistochemical staining of Bcl-2, an antiapoptotic protein. The brown color in the artery indicates the positive signal. Magnification ×400. Bar = 50 μm.*

following balloon angioplasty. On the other hand, angiotensin-converting enzyme inhibitors were ineffective in preventing restenosis (*20–22*).

In contrast to our present observations, Suzuki *et al*. (*10*) demonstrated that deletion of the AT2 receptor in mice caused exaggeration of neointimal thickening after vascular injury induced by placing a polyethylene cuff around the artery (cuff injury model). Furthermore, the neointimal thickening was associated with enhanced DNA synthesis as well as inhibition of apoptosis in neointimal cells. In the present study, the AT2 receptor appeared to result in no vascular cell proliferation *per se*, as revealed by BrdU assay. This conflicting result may be explained in part by the difference in the mechanisms of neointimal formation between the wire injury model and cuff injury model. The wire injury model is characterized by a severe and direct luminal injury associated with de-endothelialization and a rapid onset of apoptosis of medial cells after injury (*19*, *23*). On the other hand, the cuff injury produces a local inflammation (*24*) but not endothelial damage. Tanaka *et al*. (*25*) recently demonstrated that a significant number of the neointimal and medial cells were derived from bone marrow in the wire injury model, whereas bone marrow–derived cells were seldom involved in the neointima formation after cuff injury. The function of the AT2 receptor may be different in bone marrow–derived cells and vascular smooth muscle cells. The conflicting results between our study and that of Suzuki *et al*. (*10*) may also be explained by the presence or absence of endothelium in the injured lesions. The denudation of the endothelium, the major site of vascular angiotensin II formation, may have suppressed local angiotensin II generation (*26*), leading to a decrease in the contribution of AT1 and AT2 receptors to the repair and remodeling process.

We used the TUNEL assay to assess apoptosis of vascular lesions. It is known that the TUNEL assay is sensitive not only to programmed cell death but also to necrotic cell death. In the present study, the number of TUNEL-positive cells was decreased in the injured arteries of AT2KO mice (Fig. 5). This observation only indicates that stimulation of the AT2 receptor triggered vascular cell death either through apoptosis or necrosis. However, we also observed morphological changes of nuclei which were characteristic of apoptosis early

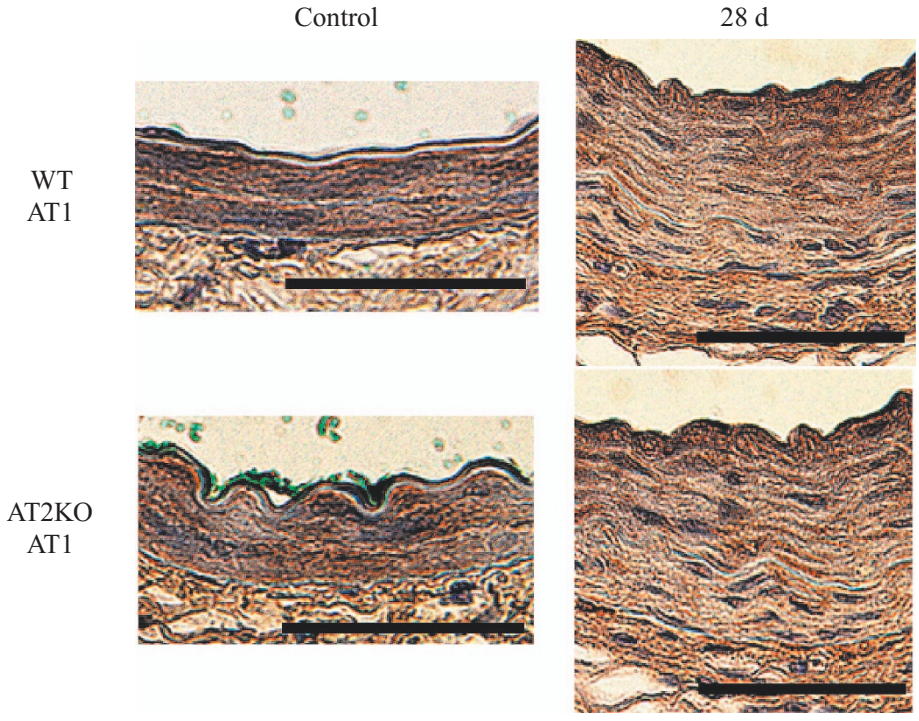

Fig. 6. *Immunohistochemical staining of the AT1 receptor in the femoral arteries of WT and AT2KO mice. The brown color in the artery indicates the positive signal. Arteries were excised from animals before (control) and 28 d after wire injury. The AT1 receptor expression level was not altered by wire injury and the staining level was similar in the two strains of animals. Magnification ×400. Bar = 50 μm.*

after injury (Fig. 5C). The expression of Bcl-2 tended to be more enhanced in AT2KO mice than in WT mice (Fig. 5D). These observations may support the notion that the increase in TUNEL-positive cells in AT2KO mice reflects enhanced apoptosis in the injured vessels. In addition, a number of studies have suggested that stimulation of the AT2 receptor is associated with induction of apoptosis (*10–14*). It has been shown that massive apoptosis of medial smooth muscle cells occurs early after vascular injury or balloon angioplasty and precedes neointimal thickening (*19*, *23*, *27*, *28*). In the present study, therefore, it is likely that vascular cell apoptosis was activated following the injury, at least in part, through stimulation of the AT2 receptor.

In summary, we demonstrated data supporting the hypothesis that the AT2 receptor has a pro-apoptotic effect on vascular cells in injured arteries. On the other hand, inhibitory action of the AT2 receptor on neointimal proliferation was not detected. Lack of the AT2 receptor caused thickening of the medial layer due to inhibition of apoptosis. However, the AT2 receptor played only a limited role in the injury-associated vascular remodeling, because the predominant feature of the remodeling was proliferation of neointimal cells associated with inhibition of apoptosis.

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