Androgen Receptor and Atherosclerosis

New Therapy via Targeting Androgen Receptor in Monocytes/Macrophages to Battle Atherosclerosis

Chuung-Kuei Huang,* Haiyan Pang,* Lin Wang, Yuanjie Niu,* Jie Luo, Eugene Chang, Janet D. Sparks, Soo Ok Lee, Chawnshang Chang

Abstract—The male sex has a higher risk to develop coronary artery diseases, including atherosclerosis. The androgen receptor (AR) is expressed in several atherosclerosis-associated cell types, including monocytes/macrophages, endothelial cells (ECs), and smooth muscle cells (SMCs), but its pathophysiological role in each cell type during the development of atherosclerotic lesions remains unclear. Using the Cre-loxP system, we selectively knocked out AR in these 3 cell types and the resultant AR knockout (ARKO) mice, monocyte/macrophage ARKO, EC-ARKO, and SMC-ARKO, were then crossed with the low-density lipoprotein receptor (LDLR) deficient (LDLR−−) mice to develop monocyte/macrophage ARKO-LDLR−−, EC-ARKO-LDLR−−, and SMC-ARKO-LDLR−− mice for the study of atherosclerosis. The results showed that the monocyte/macrophage ARKO-LDLR−− mice had reduced atherosclerosis compared with the wild-type-LDLR−− control mice. However, no significant difference was detected in EC-ARKO-LDLR−− and SMC-ARKO-LDLR−− mice compared with wild-type-LDLR−− mice, suggesting that the AR in monocytes/macrophages, and not in ECs and SMCs, plays a major role to promote atherosclerosis. Molecular mechanism dissection suggested that AR in monocytes/macrophages upregulated the tumor necrosis factor-α, integrin β2, and lectin-type oxidized LDL receptor 1 molecules that are involved in 3 major inflammation-related processes in atherosclerosis, including monocytes/macrophages migration and adhesion to human umbilical vein ECs, and subsequent foam cell formation. Targeting AR via the AR degradation enhancer, ASC-J9, in wild-type-LDLR−− mice showed similar effects as seen in monocyte/macrophage ARKO-LDLR−− mice with little influence on lipid profile. In conclusion, the AR in monocytes/macrophages plays key roles in atherosclerosis and targeting AR with ASC-J9 may represent a new potential therapeutic approach to battle atherosclerosis. (Hypertension. 2014;63:1345-1353.) ● Online Data Supplement

Key Words: androgen ■ atherosclerosis ■ macrophages ■ receptors, androgen

The male sex is one of the risk factors for coronary artery disease (CAD) and the average life expectancy for men with CAD is ≈8 years less than that of women with CAD. Atherosclerosis is the primary cause of CAD, and it therefore represents the most common cause of morbidity and mortality from CAD worldwide. Atherosclerosis is an inflammatory disease, characterized by lipid and macrophage depositions in the arterial wall. The atherosclerosis is initiated by the activation of endothelial cells (ECs), leading to high expression of adhesion molecules for recruiting inflammatory cells. Macrophage colony-stimulating factor induces monocytes to enter the plaque to differentiate into macrophages and foam cells, which is a critical step for the atherosclerosis. In the plaque centers, foam cells and extracellular lipid droplets form a core region, which is surrounded by a cap of smooth muscle cells (SMCs) and a collagen-rich matrix. T cells, macrophages, and mast cells infiltrate the lesion and are particularly abundant in the shoulder region of the plaque where the atheroma grows. Many of the immune cells exhibit signs of activation and produce inflammatory cytokines.

Almost all of the previous studies were focused on the androgen effect on atherosclerosis as androgen is considered to be the main factor for male sex risk in atherosclerosis. However, several recent results demonstrated a protective role of androgens.

Compared with the effect of androgen, few studies have been done to reveal the androgen receptor (AR) effect on atherosclerosis. AR is activated by binding of androgens, but it can also exert its function without the binding of androgen. Therefore, it becomes obvious that the AR

Received November 10, 2013; first decision December 4, 2013; revision accepted February 26, 2014.

From the George Whipple Lab for Cancer Research, Departments of Pathology, Urology, Radiation Oncology, Wilmot Cancer Center (C.-K.H., H.P., J.L.L., J.S., S.O.L., C.C.) and Department of Pathology, Aab Cardiovascular Research Institute (E.C.), University of Rochester Medical Center, Rochester, NY; Chawnshang Chang Sex Hormone Research Center, The Kidney and Blood Purification Center, Tianjin Institute of Urology, Tianjin Medical University, Tianjin, China (H.P., L.W., Y.N.); and Sex Hormone Research Center, China Medical University/Hospital, Taichung, Taiwan (C.C.).

*These authors contributed equally to this work.

The online-only Data Supplement is available with this article at http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.113.02804/-/DC1.

Correspondence to Chawnshang Chang, George Whipple Lab for Cancer Research, Departments of Pathology, Urology, Radiation Oncology, Wilmot Cancer Center, University of Rochester Medical Center, 601 Elmwood Ave, Box 626, Rochester, NY 14642. E-mail chang@urmc.rochester.edu or Lin Wang, Chawnshang Chang Sex Hormone Research Center, The Kidney and Blood Purification Center, Tianjin Institute of Urology, Tianjin Medical University, 23 Pingsjiang Road, Hexi District, Tianjin, China 300211. E-mail wang.lin@medmail.com.cn

Hypertension is available at http://hyper.ahajournals.org

DOI: 10.1161/HYPERTENSIONAHA.113.02804

Downloaded from http://hyper.ahajournals.org/ by guest on December 1, 2014
effect is not always the same as the androgen effect. One of the recent findings from wound healing studies demonstrated that the AR knockout (ARKO) effect is critical in mediating the suppressive effect in wound healing, yet such AR effects could not be reversed by androgen treatment, suggesting that AR could function through androgen-independent pathways. Ikeda et al. recently investigated the effect of androgen/AR on atherosclerosis using the apolipoprotein E–deficient (ApoE−/−) derivative of the total ARKO mice (GARKO–ApoE−/−); however, their studies failed to distinguish the effect of androgen versus AR because their mouse model lacked both AR and androgen (serum testosterone dropped to almost an undetectable level).

Based on the inflammatory characteristic of atherosclerosis and the role of AR in enhancing inflammatory responses, we hypothesized that AR would promote the progression of atherosclerosis and the AR in monocytes/macrophages might exert the primary function. In this study, cell-specific ARKO male mice were used to demonstrate that the AR in monocytes/macrophages is critical in atherosclerosis.

**Materials and Methods**

Please see the online-only Data Supplement for materials and methods.

**Results**

**Atherosclerotic Plaques Were Increased in GARKO-Low-Density Lipoprotein Receptor–Deficient Mice But Decreased in Monocytes/ Macrophage AR Knockout-Low-Density Lipoprotein Receptor–Deficient Mice Compared With the Wild-Type-Low-Density Lipoprotein Receptor–Deficient Littermate Control Mice**

Using the Cre-loxP system, we either knocked out AR ubiquitously or selectively in 3 major cell types associated with atherosclerosis: monocytes/macrophages, ECs, and SMCs, and the resultant monocytes/macrophage ARKO (MARKO), ECs–ARKO (EARKO), and SMC–ARKO (SARKO) mice were then crossed with the low-density lipoprotein receptor (LDLR)–deficient (LDLR−/−) mice to develop GARKO–LDLR−/−, MARKO–LDLR−/−, EARKO–LDLR−/−, and SARKO–LDLR−/− mice. Figure S1A in the online-only Data Supplement shows the genotyping results of 4 types of the generated ARKO mice and Figure S1B shows the depletion of AR in the bone marrow–derived macrophages obtained from MARKO–LDLR−/− mice. No significant change in body weight was observed in the MARKO–LDLR−/− mice, but we observed increases in body weights in the other 2 types of mice (EARKO–LDLR−/− and SARKO–LDLR−/−) (Figure S1C).

After atherosclerosis was developed by feeding mice with high-fat diet for 16 weeks, aortas were excised from each group of mice and stained with Oil red O to visualize atherosclerotic plaques. Compared with the wild-type (WT)–LDLR−/− littermate control mice, the GARKO–LDLR−/− showed increased atherosclerotic plaques and reduced androgen levels, which are consistent with previous findings (Figure 1A–1D). However, the MARKO–LDLR−/− mice showed significantly less atherosclerosis (25.9% reduction; P value<0.001; n=9) without an altered androgen level as compared with the WT–LDLR−/− littermate control mice (Figure 2A, 2B, and 2E). In contrast, we found little differences in plaque areas in the EARKO–LDLR−/− and SARKO–LDLR−/− mice as compared with their littermate controls. The results indicate that there are no significant differences in androgen levels among EARKO–LDLR−/−, SARKO–LDLR−/−, and their littermate controls (Figure 2A, 2B, and 2F).

We then performed hematoxylin and eosin staining of aortic tissues of these mice and detected reduced plaque area in the MARKO–LDLR−/− mice (38% reduction; P value <0.001; n=6) compared with the littermate control mice (n=5; Figure 2C; quantification of relative plaque areas is shown in Figure 2D).

Together, the results of these studies using the LDLR−/− derivatives of the 4 different ARKO mice demonstrated that the AR in monocytes/macrophages, but not in ECs and SMCs, played an important role in the promotion of atherosclerosis.

**Depletion of AR in Monocytes/Macrophages Reduces Monocytes Infiltration into the Aorta Plaque Area**

A crucial step in atherosclerosis is an infiltration of monocytes into the subendothelium of the arteries where they differentiate into macrophages and become functionally active. We therefore investigated monocytes infiltration in aortic tissues of the MARKO–LDLR−/− and WT–LDLR−/− littermate control mice by staining tissues with the monocyte-specific antibody, Mac-3. We found that the Mac-3 positively stained cell numbers (represented as positively stained cell percentage) were significantly decreased in the MARKO–LDLR−/− mice (5.28±2.11%; n=7) compared with their littermate control mice (20.57±4.42%; n=6; Figure 3A).

We also examined collagen deposition in aortas of MARKO–LDLR−/− and WT–LDLR−/− mice via Masson Trichrome staining, and results demonstrated significantly lower collagen deposition in the MARKO–LDLR−/− mice (10.14±1.08%; n=5) compared with the littermate control mice (14.48±1.28%; n=5; Figure 3B).

We further investigated whether invaded SMC numbers were modulated by the ARKO in monocytes/macrophages and found that the α-smooth muscle actin positively stained SMC numbers were increased in the MARKO–LDLR−/− mice (28.70±1.94%; n=5) compared with the control littermate mice (12.37±1.71%; n=6; Figure 3C). This suggested that higher numbers of the migrated SMCs may stabilize the plaques leaving them less susceptible to rupture. Therefore, we speculate that the ARKO in monocytes/macrophages might contribute to the stability of plaques as shown by the increased numbers of the invaded SMCs in MARKO–LDLR−/− mice, thereby protecting mice from developing into the acute coronary syndrome.

Together, results of Figure 3A to 3C demonstrate that the loss of AR in monocytes/macrophages resulted in decreased infiltrating monocytes/macrophages in the subendothelium of arteries with decreased collagen deposition and increased SMCs, and all these combined effects led to the suppression of atherosclerosis.

**AR in Monocyte THP-1 Cells Promoted Their Migration and Adhesion to Human Umbilical Vein ECs and Foam Cell Formation**

To further confirm the above in vivo findings, we applied in vitro assays to show the AR role in monocytes/macrophages
in promoting atherosclerosis. We first investigated whether the AR expression in monocytes influences their migration. Monocyte THP-1 cells were infected with lentivirus carrying either scramble control sequence or AR-siRNA and Figure 4A-a (upper) shows successful knockdown of AR in these cells. In the migration assay, the THP-1 cells, either the AR knocked down (THP-1siAR) or the scramble control (THP-1sc) cells, were placed in the upper chamber, whereas the conditioned media of human umbilical vein ECs (HUVECs) were placed in the lower chamber of transwell plates as shown in Figure 4A-a (lower) cartoon. We found that the migration of the THP-1siAR cells was suppressed, when compared with the THP-1sc cells (Figure 4A-b). However, when we manipulated AR expression in HUVECs and used the conditioned media of the AR expressing and depleted HUVECs in the tests, migration of THP-1 cells was not influenced significantly (data not shown), suggesting that AR expression in monocytes, and not in ECs, is critical in mediating this migration process.

We also tested adhesion of THP-1 cells onto HUVECs (a cartoon in Figure 4A-c, upper). Similarly, we used THP-1siAR and THP-1sc cells in the test and tagged THP-1 cells with green fluorescence dye before the reaction so that the adhered green fluorescence dye–labeled cells can easily be detected (middle). After incubation of 2 types of cells for 2 hours at 37°C, numbers of the adhered fluorescent THP-1 cells were counted. Similar to the migration test, we found that adhesion of the THP-1siAR onto HUVECs was significantly reduced (60%) compared with the THP-1sc (Figure 4A-c, lower; quantification, right; representative images in Figure S2A). However, the manipulation of AR expression in HUVECs did not influence adhesion of THP-1 cells onto HUVECs significantly (data not shown). These results also suggest that AR expression in monocytes, and not in ECs, is critical in mediating this migration process and in promoting the adhesion process.

In addition to migration and adhesion, foam cell formation is the third key step of atherosclerosis development. Therefore, we tested whether the AR expression in THP-1 cells can also affect this process. THP-1siAR and THP-1sc cells were treated with mouse-derived colony-stimulating factor to stimulate differentiation of monocytes into macrophages and these resultant macrophages were subsequently treated with oxidized LDL to induce foam cell formation. After staining of foam cells with Oil red O, the positively stained cells numbers were compared. As shown in Figure 4A-d, the THP-1siAR cells have significantly reduced foam cell formation (11.89±2.18%) as compared with the THP-1sc control cells (17.65±1.72%), indicating that the AR in THP-1 cells also plays a stimulatory role in this process.

Taken together, Figure 4A results suggest that the AR in monocytes/macrophages plays positive roles in promoting the 3 major inflammation-associated processes in atherosclerosis: (1) migration, (2) adhesion of monocytes to ECs, and (3) subsequent foam cell formation.

**Key AR-Modulated Molecules That Affect Migration of Monocytes to ECs in THP-1 Cells**

We next investigated the molecular mechanisms by which the AR in monocytes/macrophages promote the 3 inflammation-associated processes via modulation of expressions of the target molecules of these processes.

Among the migration-related molecules, including tumor necrosis factor (TNF-α), interleukin-6, chemokine (C-C motif) ligand 2 and C-C chemokine receptor type 2 we assayed, we found that the mRNA expressions of TNF-α and interleukin-6 were the most significantly inhibited in THP-1siAR cells compared with those in THP-1sc cells (Figure 4B-a). We then selected TNF-α for further investigation. As shown in Figure 4B-b, we found that the secreted TNF-α protein was decreased dramatically in THP-1siAR cells compared with that in the THP-1sc cells, confirming the positive regulation of AR in TNF-α expression at both protein and mRNA levels.

We then asked whether AR could modulate TNF-α at transcriptional levels via chromatin immunoprecipitation assay. The results showed that the AR could bind to the androgen response element (ARE; TNF promoter ARE1: +36/+41; ARE2: +50/+55) on the 5′ promoter region of TNF-α (Figure 4B-c), and the luciferase assay also confirmed that AR could induce TNF-α expression at the transcriptional level (Figure 4B-d).

We further applied functional assays to test whether blocking TNF-α via a neutralizing antibody of TNF-α could suppress the AR effect in promoting migration of THP-1 cells into ECs. As shown in Figure 4B-e, addition of TNF-α neutralizing

---

**Figure 1.** Plaque formation was accelerated in general androgen receptor knockout in low-density lipoprotein receptor–deficient (GARKO-LDLR−/−) mice compared with the wild-type (WT)-LDLR−/− littermate control mice. **A**, Plaque area was analyzed after staining with Oil red O stain. Graph on the right is quantification results of plaque area over total area. **B**, Masson Trichrome staining was used to analyze collagen deposition in WT-LDLR−/− and GARKO-LDLR−/− mice. Graph on right shows the quantification results of collagen positive area over total area. Arrows indicate collagen area (blue color). Magnification, ×400. Androgens, **C** testosterone, and **D** dihydrotestosterone (DHT) were measured using ELISA kit in WT-LDLR−/− and GARKO-LDLR−/− mice. *P*<0.05, **P*<0.01, and ***P*<0.001.

---

**Huang et al** 

**Macrophage AR Promotes Atherosclerosis**
antibody reduces THP-1sc cells migration to the level of the THP-1siAR cells, indicating that TNF-α is indeed critical in mediating the promoter role of AR in this process.

We also examined whether the TNF-α expression is reduced in the aortic tissues of the MARKO-LDLR−/− mice compared with their WT-LDLR−/− littermate control mice and found significantly reduced expressions of TNF-α in aortic tissues of MARKO-LDLR−/− mice compared with the WT-LDLR−/− littermate control mice (Figure 4B-f), confirming the in vitro effect of AR modulation of TNF-α for promoting monocytes/macrophages migration to ECs.

**Key AR-Modulated Molecules That Affect Adhesion of Monocytes to ECs in THP-1 Cells**

Next we surveyed molecules that have been documented to play important roles in the adhesion process,15,16 and found that the expression of integrin family members (integrin [ITG] αM, αV, β1, β2) and P-selectin glycoprotein ligand was suppressed in THP-1siAR cells compared with that in the THP-1sc control cells (Figure 4C-a). We further dissected the molecular mechanism how AR could modulate ITGβ2 in the adhesion process, and confirmed AR could modulate ITGβ2 expression at both mRNA and protein levels (Figure 4C-a and b).
β2 in vivo. We also confirmed the AR-mediated reduction of ITGβ2 expression in the aortic tissues of the MARKO-LDLR−/− mice and their WT-LDLR−/− littermate control mice (Figure 4C-f), which compared with the wild-type (WT)-LDLR−/− mice. WT-LDLR−/− mice stained with α-smooth muscle actin (SMA). Magnification, ×100. Quantification is shown below images. **P<0.01.

We then investigated the target molecules in the foam cell formation process. The initial studies on wound healing demonstrated that ASC-J9 was able to selectively degrade AR in monocytes/macrophages and mimicked the MARKO-LDLR−/− mice effect and subsequent foam cell formation. Our results showed that ASC-J9 could enhance AR protein degradation in THP-1 cells and subsequent foam cell formation. Hence, we continued to investigate the AR-mediated reduction of ITGβ2 expression in aortic tissues of MARKO-LDLR−/− mice compared with their littermate control mice (Figure 4C-f), which also confirmed the AR-mediated reduction of ITGβ2 in vivo.

Key AR-Modulated Molecules That Affect Foam Cell Formation in THP-1 Cells

We then investigated the target molecules in the foam cell formation process, such as lectin-type oxidized LDL receptor 1 (LOX-1), lysosomal acid lipase, and acyl–coenzyme A: cholesterol acyltransferase, in THP-1siAR and THP-1sc cells and found that expressions of these 3 molecules were suppressed in the THP-1siAR cells (Figure 4D-a). Further analysis of LOX-1 expression in THP-1siAR and THP-1sc cells showed lower mRNA expression of LOX-1 in THP-1siAR cells compared with the THP-1sc cells (Figure 4D-b).

We further tested whether the blocking of LOX-1 by treating the THP-1siAR and THP-1sc cells with the LOX-1 neutralizing antibody could inhibit foam cell formation. As shown in Figure 4D-e, the foam cell formation was significantly inhibited on antibody treatment in THP-1sc cells, but not in THP-1siAR cells.

We then examined LOX-1 expression in the aortic tissues of the MARKO-LDLR−/− and their WT-LDLR−/− littermate control mice and found significantly reduced expressions of LOX-1 in aortic tissues of MARKO-LDLR−/− mice compared with their littermate control mice (Figure 4D-f), suggesting that AR could go through modulation of LOX-1 expression to influence form cell formation in vivo.

Together, results from Figure 4 show that the AR in monocytes/macrophages might play a stimulatory role to promote atherosclerosis via multiple modulations of various key molecules, which might explain why we could detect decreased plaque formation in the MARKO-LDLR−/− mice (see Figure 2).

Targeting Monocyte/Macrophage AR With ASC-J9 to Battle Atherosclerosis

We then asked how we can target monocytes/macrophages AR in the body to mimic the MARKO-LDLR−/− mice effect and use that strategy as a potential new therapeutic approach to battle atherosclerosis. ASC-J9 is an AR degradation enhancer, which functions through the proteasome machinery to selectively degrade AR. ASC-J9 could increase Akt-modulated AR and Mdm2 interaction and recruit the proteasome complex to enhance AR’s degradation.18 The earlier studies on wound healing demonstrated that ASC-J9 was able to selectively degrade AR in monocytes/macrophages and mimicked the MARKO mice effect with improved wound healing.11

We first tested the in vitro ASC-J9 effects in inhibiting 3 processes: migration and adhesion of monocytes onto ECs and subsequent foam cell formation. Our results showed that ASC-J9 could enhance AR protein degradation in THP-1 cells starting at 2.5 μmol/L concentration (Figure 5A). When we used 5-μmol/L ASC-J9 to test its effect on THP-1 cell migration into HUVECs, we found significant reduction in THP-1 cells migration (Figure 5B). We then tested ASC-J9 effect on the adhesion of THP-1 cells onto HUVECs. As shown in Figure 5C, THP-1 cell adhesion was also significantly reduced on ASC-J9 treatment (representative images in Figure S2C). We further examined ASC-J9 effects on foam cell formation...
Figure 4. A, Androgen receptor (AR) in THP-1 cells play a stimulatory role to promote the migration and adhesion to human umbilical vein endothelial cells (HUVECs) and foam cell formation. **P<0.01. b, THP-1siAR and THP-1sc were tagged with green fluorescence dye and then incubated with HUVECs. The adhered THP-1 cells with green fluorescence were counted. Quantification is shown on the right. Magnification, x100. **P<0.01. c, The THP-1siAR and THP-1sc cells were induced to differentiate into macrophages by mouse-derived colony-stimulating factor (M-CSF). The cells were then incubated with oxidized low-density lipoprotein (oxLDL) to evaluate the ability of cells to uptake oxLDL (cartoon at left). The end of reaction, cells were stained with filtered Oil red O (middle). Magnification, x100. Quantification is shown on the right. **P<0.01. B, The AR in THP-1 cells promotes migration to HUVECs. a, Quantitative polymerase chain reaction (qPCR) analysis testing mRNA expression levels of candidate molecules involved in migration process. **P<0.01. b, ELISA test showing tumor necrosis factor (TNF)-α secretion by the THP-1siAR and THP-1sc cells. ***P<0.001. c, Chromatin immunoprecipitation (ChIP) assay. AR antibody was used to pull down TNF-α promoter region, which contains androgen response elements (AREs; upper). d, TNF-α promoter transactivation was measured using luciferase constructs containing AR binding sites of the promoter region of TNF-α molecule in the presence of pBabe vector only or pBabe-AR. Human embryonic kidney (HEK)-293 cells were used in this assay. e, Blocking effect of migration of THP-1 cells to HUVECs on incubation with the TNF-α antibody. f, Immunohistochemical (IHC) staining of TNF-α in aortic tissues obtained from the monocyte/macrophage AR knockout (MARKO-LDLR−/−) and wild-type (WT)-LDLR−/− mice. Arrowheads indicate positive stained areas. Magnification, x100. Quantification is shown on the right. **P<0.01, ***P<0.001. C, The AR in THP-1 cells promotes adhesion to HUVECs. a, qPCR analysis testing mRNA expression levels of candidate molecules involved in adhesion process. b, Western blot analysis showing AR and integrin (ITG) β2 in the THP-1siAR and THP-1sc cells. GAPDH was used as control. c, ChIP assay. AR antibody was used to pull down ITGβ2 promoter region, which contains AREs. d, ITGβ2 promoter transactivation was measured using luciferase construct containing ITGβ2 promoter region in the presence or absence of AR with or without dihydrotestosterone (DHT). HEK-293 cells were used in this assay. e, Blocking effect of adhesion of THP-1siAR and THP-1sc cells onto the HUVECs by the ITGβ2 antibody. f, IHC staining of ITGβ2 in aortic tissues obtained from the MARKO-LDLR−/− and WT-LDLR−/− mice. Magnification, x400. Arrowheads indicate positive stained area. Quantification is shown on the right. **P<0.01, ***P<0.001. D, The AR in macrophages promotes foam cell formation. a, qPCR analysis testing mRNA expression levels of candidate molecules involved in foam cell formation process. **P<0.01. b, AR and lectin-type oxidized LDL receptor 1 (LOX-1) expression levels were determined using Western blot in THP-1sc and THP-1siAR cells. GAPDH served as loading control. c, LOX-1 promoter region containing ARE (upper) was pulled down with AR antibody and amplified with primers specific targeting ARE region (lower). d, LOX-1 promoter region was constructed to pGL3 luciferase vector. The LOX-1 promoter transactivation was measured in the presence or absence of AR with or without DHT. e, Blocking effect on foam cell formation on incubation with the LOX-1 antibody. Quantification is shown on the right. f, IHC staining of LOX-1 in aortic tissues obtained from the MARKO-LDLR−/− and WT-LDLR−/− mice. Magnification, x100. Quantification is shown on the right. **P<0.01, ***P<0.001.
We then extended our studies of ASC-J9 effects into in vivo mice. ASC-J9 was injected intraperitoneally every other day (75 mg/kg per day) into the 16-week-old WT-LDLR−/− mice, 8 weeks after starting high-fat diet feeding and continued for 8 weeks. In parallel, control littermates received vehicle only. After a total of 16 weeks of high-fat diet feeding, mice were euthanized and atherosclerosis parameters were analyzed. Interestingly, the results showed that the WT-LDLR−/− mice treated with ASC-J9 had significantly decreased plaque area (25.1±4.9%; n=11) compared with the vehicle control mice (19.2±5.7%; n=5; Figure 6A). We then examined whether the treatment with ASC-J9 leads to decreased AR expression in macrophages. The immunohistochemical staining revealed that expression of the monocyte/macrophage AR (Figure 6B) and macrophages infiltration (Figure 6C) were significantly decreased in these ASC-J9–treated mice. Importantly, we found that mice treated with ASC-J9 still had normal body weights with near normal serum testosterone and normal fertility, which is consistent with the previous results showing few side effects.11,19

Together, the results from in vitro cell lines (Figure 5) and in vivo mice studies (Figure 6) suggest that ASC-J9 may have a therapeutic potential to battle the atherosclerosis in the future.

Discussion

In these studies we found that general ARKO in LDLR−/− (GARKO-LDLR−/−) mice significantly increased atherosclerotic plaques, whereas ARKO in monocytes/macrophages significantly reduced plaque area as shown in MARKO-LDLR−/− mice. However, the mice lacking AR in ECs and SMCs failed to show such an effect, suggesting that the AR in monocytes/macrophages may play critical roles in promoting atherosclerosis. Interestingly, MARKO-LDLR−/− mice showed completely opposite results to the GARKO-LDLR−/− mice. Actually, there may be multiple factors contributing to these contrasting effects observed in GARKO-LDLR−/− versus MARKO-LDLR−/− mice on atherosclerosis. First, this may be explained by the serum testosterone level difference because the serum testosterone levels in the GARKO-LDLR−/− mice dropped dramatically, whereas the serum testosterone levels in the MARKO-LDLR−/− mice remained almost unchanged as compared with the WT-LDLR−/− littermate control mice. Early studies documented that testosterone therapy could lead to reduced cholesterol and LDL levels,20–23 and higher frequency of atherosclerosis was also observed in prostate cancer patients after receiving androgen deprivation therapy,24 suggesting that androgens may play protective roles in atherosclerosis development. Our results showing GARKO-LDLR−/− mice with low level of serum testosterone developed atherosclerosis, yet MARKO-LDLR−/− mice with normal serum testosterone level developed little atherosclerosis are in agreement with these clinical data.

Second, we may explain the opposite atherosclerosis phenotypes of the GARKO-LDLR−/− and MARKO-LDLR−/− mice by lipid profile differences. When we investigated lipid levels in these 2 types of ARKO mice, it was shown that total cholesterol and triglyceride contents were significantly higher in GARKO-LDLR−/− mice than in the WT-LDLR−/− littermate control mice, whereas MARKO-LDLR−/− mice showed little change (Figure S3A and S3B). These results suggest that the higher levels of cholesterol and triglyceride in GARKO-LDLR−/− mice might contribute to the higher rate of plaque formation.

The high lipid levels in GARKO-LDLR−/− mice may be because of the low testosterone levels. However, our earlier studies in GARKO mice of metabolism and the current findings in SARKO-LDLR−/− mice indicate that the higher lipid levels may not be independently affected by testosterone levels.25 When we investigated whole lipid/glucose metabolism...
and visceral obesity. Therefore, it can be speculated that the neuronal ARKO in neurons in the neuronal ARKO mice resulted in significant increases in triglyceride and cholesterol with-elevating lipid levels. We found that ARKO in SMCs results in higher lipid levels, and these high lipid levels were not noticeable between GARKO mice, we observed that GARKO mice had high lipid levels, and these high lipid levels were not reversed when we treated the GARKO mice with androgen. Next, we investigated in which tissues the ARKO is critical in elevating lipid levels. We found that ARKO in SMCs results in significant increases in triglyceride and cholesterol without altering androgen levels (Figure S3C). We also found that the ARKO in neurons in the neuronal ARKO mice resulted in high lipid levels and further revealed that the neuronal ARKO mice had induced hypothalamic insulin resistance, which, in turn, led to hepatic insulin resistance, lipid accumulation, and visceral obesity. Therefore, it can be speculated that the higher lipid levels observed in GARKO-LDLR−/− mice may be related to the smooth muscle cellular and neuronal ARKO in those mice. Although SARKO-LDLR−/− mice exhibit similar atherosclerotic plaques as the littermate controls, whether the atherosclerosis mouse model using the neuronal ARKO mice will lead to higher atherosclerosis similar to the GARKO-LDLR−/− mice needs to be investigated in the future to draw a solid conclusion.

No matter what the major factor is, it is obvious that targeting androgen/AR signaling in the whole body (as current androgen deprivation therapy does in prostate cancer patients using various antiandrogens to suppress androgen binding to AR in the whole body) may not be an effective way to suppress atherosclerosis and therefore, we need to develop a way to suppress monocytes/macrophages without affecting testosterone and lipid levels.

We, therefore, suggest the use of the AR degradation enhancer ASC-J9 as a new potential therapeutic approach to battle atherosclerosis with rationales as follows. (1) The ASC-J9–treated WT-LDLR+ mice mimicked the MARKO-LDLR− mice effect, similar to the early study demonstrating that ASC-J9 could degrade AR in macrophages to reach the therapeutic effect in macrophages-mediated wound healing. (2) The ASC-J9–treated WT-LDLR−/− mice showed reduced plaque formation to a similar extent as observed in MARKO-LDLR−/− mice. (3) Mice treated with ASC-J9 show little change in serum testosterone and AR could be degraded in selective cell types, including macrophages, SMCs, prostate, and heart (Figure S4A). (4) In vivo data demonstrated that ASC-J9–treated mice have normal libido and sexual activity with normal fertility.

In summary, we conclude that targeting AR in monocytes/macrophages may be a new effective therapy to battle atherosclerosis that can be achieved by exploiting ASC-J9 as a therapeutic approach. Figure S5 cartoon explains our entire approach.

**Perspectives**

Atherosclerosis is an inflammatory and metabolic disease. AR plays important roles in metabolism and inflammation. AR promotes inflammatory responses as demonstrated using castrated male mice and GARKO mice to show reduced inflammatory responses. However, androgen and AR signaling are complicated in lipid and glucose metabolism and abnormal metabolism would increase lipid accumulation in the blood stream to cause atherosclerosis. In our previous studies, we have shown that neuron and hepatocyte-specific ARKO mice have elevated lipid profiles, whereas adipocyte-specific ARKO mice have decreased lipid profiles. In the current study, we showed that MARKO mice have slightly lower lipid profiles, EARKO mice have no change on lipid profiles, but SARKO mice have surprisingly elevated lipid profiles, even higher than GARKO mice. From these results of many cell-specific ARKO mice, it could be speculated that the changed lipid profiles of GARKO mice is a summation of AR effects on different kinds of cells that are involved in metabolism. It might not be easy to simply target AR in the whole body, because this would worsen the atherosclerosis progression. Luckily, we investigated an AR degradation enhancer, ASC-J9, and found that it has the potential to target AR in monocytes/macrophages to suppress atherosclerosis. Although ASC-J9 could suppress atherosclerosis progression, we actually do not know whether treatment with ASC-J9 could result in insulin resistance, which has been shown using GARKO and several cell-specific ARKO mice. Future studies are needed to evaluate the safety of ASC-J9 before initiating future clinical studies.

**Acknowledgments**

We thank K. Wolf for help in editing the article.

**Source of Funding**

This work was supported by National Institutes of Health grants (CA127300 and CA156700) and Taiwan Department of Health Clinical Trial and Research Center of Excellence grant DOH99-TD-B-111-004 (China Medical University, Taichung, Taiwan).
Disclosures
ASC-J9 was patented by the University of Rochester, the University of North Carolina, and AndroScience, and then licensed to AndroScience. Both the University of Rochester and C. Chang own royalties and equity in AndroScience. The other authors report no conflicts.

References

Novelty and Significance

What Is New?
• This is the first study using cell-specific androgen receptor (AR) knockout mice to further clarify AR roles in 3 major types of cells that are involved in atherosclerosis initiation and progression.

What Is Relevant?
• AR in monocytes/macrophages is important for atherosclerosis development but not AR in endothelial cells and smooth muscle cell. Although general AR knockout mice develop severe atherosclerosis, monocytes/macrophage AR knockout mice have reduced atherosclerotic plaques, indicating that targeting AR in monocytes/macrophages might have therapeutic effects on atherosclerosis. Using an AR degradation enhancer, ASC-J9, to treat atherosclerotic mouse, we were able to show that ASC-J9 might be a potential therapeutic approach in atherosclerosis.

Summary
Monocytes/macrophages AR would promote atherosclerosis progression. Targeting monocytes/macrophages AR with ASC-J9, an AR degradation enhancer could suppress atherosclerosis progression and suggested targeting monocytes/macrophages AR as a novel therapeutic approach in atherosclerosis.
ONLINE DATA SUPPLEMENT:

New therapy via targeting androgen receptor in monocytes/macrophages to battle atherosclerosis

Chiung-Kuei Huanga,*, Haiyan Pangb,a,*, Yuanjie Niub, Jie Luoa, Lin Wangb, Eugene Changc, Janet Sparksa, Soo Ok Leea and Chawnshang Changa,d,#

agaGeorge Whipple Lab for Cancer Research, Departments of Pathology, Urology, Radiation Oncology, and the Wilmot Cancer Center, University of Rochester Medical Center, NY 14642, USA

bChawnshang Chang Sex Hormone Research Center and The Kidney and Blood Purification Center, Tianjin Institute of Urology, Tianjin Medical University, Tianjin 300211, China

cAab Cardiovascular Research Institute and Department of Pathology, University of Rochester Medical Center, NY 14642, USA

dSex Hormone Research Center, China Medical University/Hospital, Taichung 404, Taiwan

(*Contributed equally)

#Corresponding Authors: Chawnshang Chang

E-mail: chang@urmc.rochester.edu

Phone: 1-585-273-4500

Fax: 1-585-756-4133

Running Title: Macrophage AR promotes atherosclerosis
Supplemental Material and Methods

Animals
All mice used in developing transgenic mice were of the C57BL/6 background. The floxed androgen receptor (fAR) mice were generated by inserting loxP sites to flank of exon 2 of AR gene. The LDLR-/-, β-actin promoter driven Cre (ACTB Cre) (for GARKO), lysozyme promoter driven Cre (Lyz-Cre) (for MARKO), vascular endothelial cadherin promoter driven Cre (VEcad-Cre) (for EARKO), and transgelin promoter drive (Tagln-Cre) (for SARKO) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The fAR/AR-LDLR-/- mice and the Cre+/- LDLR-/- mice were generated by crossing the fAR/AR or the Cre+/- mice with LDLR-/- mice separately. Subsequent crossing of the resultant fAR/AR-LDLR-/- mice with the Cre+/- LDLR-/- mice led to the generation of double KO, cell specific ARKO-LDLR-/- (MARKO-LDLR-/-, EARKO-LDLR-/- AND SARKO-LDLR-/-). Accelerated atherosclerosis was induced by feeding mice with high fat diet (HFD, containing 1.25% cholesterol) for 16 weeks under a 12-hour light/12-hour dark cycle, starting at 8 weeks of age. All animal protocols were approved by the University of Rochester Committee on Animal Resources.

Cell lines and reagents
THP1 and HUVEC cells were purchased from ATCC TNFα ELISA kit was purchased from eBioscience. Manson Trichrome staining kit was purchased from Sigma. Oil-red-O powder was purchased from Sigma. High fat diets (HFD) were purchased from Teklad Lab Animal Diets-Harlan Laboratories.

ASC-J9® treatment of mice
ASC-J9® was provided by AndroScience Inc. (CA). ASC-J9® (75 mg/Kg of body weight) was suspended in dimethylacetamide (DMA), diluted with saline, and given to mice every day (i.p) 8 wks after starting HFD feeding and continued for 8 wks. In parallel, control littermates received vehicle only.

Atherosclerotic lesion analysis
After 16 weeks of HFD feeding, mice were sacrificed with peritoneal overdose injection of pentobarbital. Whole aortas were excised and thoroughly cleaned by removing adventitial fat and connective tissue under microscope. Then the aortas were dissected and opened longitudinally, fixed in 4% paraformaldehyde for 1 hr, and stained with Oil-red-O solution for 1 hr. Aortas were then rinsed with distilled water and images acquired with microscope. The percentage of plaque area to total aortic area was quantified using Image Pro Plus 5.0 software. 10 µm paraffin sections were prepared for examining cross section plaque area (by H&E staining), and collagen deposition (by Masson’s Trichrome Staining). For immunohistostaining (IHC) and immunofluorescence (IF) staining, paraffin sections were incubated with primary antibodies against AR (N20, Santa Cruz), Mac-3 (dilution 1:250 BD Biosciences), alpha smooth muscle actin (SMA) (dilution 1:250 Sigma-Aldrich), integrin (ITG), and lectin-type oxidized LDL receptor 1 (LOX-1), followed by incubation with the secondary antibodies conjugated with either 3, 3′-diaminobenzidine (DAB) for IHC, or Alexa Fluor 488 or 596 for IF, and DAPI for nuclear staining. The sections were examined under microscope (BX41; Olympus) and the positive stained areas (cells) were quantified by Image Pro Plus 5.0 software.

Analysis of levels of lipids and androgens in sera of mice obtained
Mice blood samples were collected after 16 hrs of fasting. Sera were obtained by
centrifugation and stored at -80°C for further analysis. Serum concentrations of total cholesterol (Cayman), triglycerides (Thermo Scientific), HDL, LDL (Bioassay Systems), testosterone (Endocrine Technologies) and DHT (Alpha Diagnostic International) were determined according to the instructions given by the companies indicated.

Cell culture and transfection.

HUVECs cells were cultured in HUVEC media (NIH) supplemented with EC growth kit including human recombinant VEGF, EGF, FGF, and IGF-1, L-glutamine, heparin sulfate, hydrocortisone hemisuccinate, fetal bovine serum, ascorbic acid (ATCC). THP-1 cells were cultured in RPMI-1640 with 10% serum, 1% L-glutamine, 1% penicillin-streptomycin. The 293T cells were transfected using pWPI lentiviral vectors carrying AR-siRNA or its scramble siRNA control using Lipofectamine 2000 (Invitrogen). After 4 hrs of transfection, media were replaced with the complete media and the supernatants were collected. THP-1 cells were then infected with the supernatants.

Transwell migration assay

Migration of THP-1 cells was tested using transwell plate with polycarbonate filters (5 µm pores). THP-1 cells, infected with lentivirus carrying either AR-siRNA or scrambled siRNA, or treated with ASC-J9®, were placed into the upper wells, whereas the conditioned medium (CM) of HUVECs were placed in lower chamber. The migration of THP-1 cells into the HUVECs CM was then determined after 24 hrs of incubation. The migrated cells were stained and counted under a microscope.

Adhesion assay

THP-1 cells were incubated with green fluorescence labeling dye (Molecular Probes, Eugene, OR) for half hour and used for reaction. The same numbers of HUVECs and the GFP-tagged THP-1 cells, THP-1siAR and THP-1sc, were placed in the culture dishes in the presence of amixture of the HUVEC and THP-1 cell culture medium (1:1) and incubated for 2 hrs at 37°C to allow adhesion of THP-1 cells to HUVECs. The unbound cells were washed away with PBS 3 times gently and the number of adhered THP-1 cells with green fluorescence was counted under microscope.

Foam cell formation assay

The THP-1 cells, THP-1siAR and THP-1sc, were induced to differentiate into macrophages by M-CSF (R & D Systems) for 3 days. Then the cells were incubated with oxLDL at 37°C for 24 hrs to evaluate the ability of cells to uptake oxLDL. Then the cells were fixed with 4% paraformaldehyde for 30 min and stained with filtered Oil-red-O. To test the effect of ASC-J9® on foam cell formation, cells were treated with ASC-J9® for 2 days, and then followed by incubation with M-CSF. The number of Oil-red-O positively stained foam cells was counted under a microscope.

Real-time quantitative PCR analysis

Total RNAs were obtained from THP-1 cells, infected with lentivirus carrying either AR-siRNA or scrambled RNA using Trizol reagent (Invitrogen). RNAs (5 µg) was used for reverse transcription reaction using SuperScript III Reverse Transcriptase (Invitrogen). The cDNAs obtained were subjected to real-time PCR for detecting mRNA level of AR, TNF-α, IL-6, ITG, and Lox-1 using SYBR Green (Invitrogen) and iCycler system (BioRad).
Masson’s Trichrome Staining
Masson’s Trichrome staining was performed using Masson’s Trichrome kit (Sigma-Aldrich) according to the instruction manual.

Statistical analysis
Quantitative data are expressed as mean ± SD. 2-tailed Student’s t test or 1-way ANOVA were used for comparisons of parameters among two groups. One-way analysis of variance was used for three group parameters comparisons. The value of $p < 0.05$ was considered statistically significant. *$p<0.05$, **$p<0.01$, and ***$p<0.001$
S 1. The genotyping of mice models. a. The genotypes of fAR-LDLR and Cre in WT-LDLR-/-, GARKO-LDLR-/-, MARKO-LDLR-/-, EARKO-LDLR-/-, and SARKO-LDLR-/-. b. AR proteins were detected in bone marrow macrophages (BM-MSCs) of WT-LDLR/- and MARKO-LDLR/- mice. c. Body weights of 4 kinds of specific ARKO-LDLR/- mice. *p<0.05 and ***p<0.001
S 2. THP1 cells adhesion studies. (a) Representative images of Fig. 4A-c. (b) Representative images of Fig. 4C-e. (c) Representative images of Fig. 5c.
S 3. Analysis of triglyceride, HDL, and LDL levels in sera of the GARKO-LDLR-/-, MARKO-LDLR-/-, EARKO-LDLR-/-, SARKO-LDLR-/-, and WT-LDLR-/- mice. 

(a) Lipid profile analysis results of WT-LDLR-/- and GARKO-LDLR-/- mice. 

(b) Lipid profiles of the MARKO-LDLR-/- together with their WT-LDLR-/- littermate control mice. 

(c) Lipid profile analysis results of EARKO-LDLR-/- and SARKO-LDLR-/- together with their WT-LDLR-/- controls. *p<0.05 and *** p<0.0001
**S 4.**

**a.** Western blot analysis demonstrating AR levels in several organs of the ASCJ-9® treated WT-LDLR/- and the vehicle treated control mice. SV: Seminal Vesicle.  

**b.** IHC staining of AR in plaque areas of aortas from vehicle and ASC-J9® treated mice. Ellipse dotted lines indicate smooth muscle layers. Arrowheads indicate infiltrated cells.

<table>
<thead>
<tr>
<th></th>
<th>Prostate</th>
<th>Testis</th>
<th>Liver</th>
<th>Kidney</th>
<th>Muscle</th>
<th>SV</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASC-J9</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>AR</td>
<td><img src="image1.png" alt="Western Blot Image" /></td>
<td><img src="image2.png" alt="Western Blot Image" /></td>
<td><img src="image3.png" alt="Western Blot Image" /></td>
<td><img src="image4.png" alt="Western Blot Image" /></td>
<td><img src="image5.png" alt="Western Blot Image" /></td>
<td><img src="image6.png" alt="Western Blot Image" /></td>
</tr>
<tr>
<td>GAPDH</td>
<td><img src="image7.png" alt="Western Blot Image" /></td>
<td><img src="image8.png" alt="Western Blot Image" /></td>
<td><img src="image9.png" alt="Western Blot Image" /></td>
<td><img src="image10.png" alt="Western Blot Image" /></td>
<td><img src="image11.png" alt="Western Blot Image" /></td>
<td><img src="image12.png" alt="Western Blot Image" /></td>
</tr>
</tbody>
</table>

Vehicle | ASC-J9
S 5. A cartoon showing our entire approach.
New Therapy via Targeting Androgen Receptor in Monocytes/Macrophages to Battle Atherosclerosis

Chiung-Kuei Huang, Haiyan Pang, Lin Wang, Yuanjie Niu, Jie Luo, Eugene Chang, Janet D. Sparks, Soo Ok Lee and Chawnshang Chang

_Hypertension_. 2014;63:1345-1353; originally published online March 31, 2014;
doi: 10.1161/HYPERTENSIONAHA.113.02804

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/63/6/1345

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2014/03/31/HYPERTENSIONAHA.113.02804.DC1.html

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Hypertension_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Hypertension_ is online at:
http://hyper.ahajournals.org/subscriptions/

Downloaded from http://hyper.ahajournals.org/ by guest on December 1, 2014