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MDM2: A Novel Mineralocorticoid-Responsive Gene Involved in Aldosterone-Induced Human Vascular Structural Remodeling

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Aldosterone has been demonstrated to play an important role in the pathogenesis of various cardiovascular diseases. Vascular structural remodeling, including vascular smooth muscle cell (VSMC) proliferation, has been also reported in small resistance arteries of patients with primary aldosteronism. Therefore, in this study, we examined whether genes involved in the regulation of the cell cycle were induced by aldosterone alone in cultured human VSMCs and in human small resistance arteries. Results of these studies eventually demonstrated that *MDM2*, one of the genes involved in anti-apoptosis and cell growth, was markedly increased in mineralocorticoid receptor (MR)-positive VSMCs by aldosterone in all microarray, reverse transcriptase-polymerase chain reaction, immunoblotting, and immunofluorescence analyses. In addition, an analysis using small interfering RNA demonstrated that this gene product was involved in cell proliferation of VSMCs induced by aldosterone. Eplerenone, a specific MR antagonist, inhibited this gene induction by aldosterone in VSMCs. *MDM2* protein was also more abundant in VSMCs of small resistance arteries in patients with primary aldosteronism compared with a control population. *MDM2* is therefore considered one of the mineralocorticoid-responsive genes that regulates cell proliferation of VSMCs induced by MR-mediated aldosterone stimulation, possibly playing an important role in aldosterone-induced vascular structural remodeling. (Am J Pathol 2006, 169:362–371; DOI: 10.2353/ajpath.2006.051351)

Aldosterone is a steroid hormone synthesized in the zona glomerulosa of human adrenal cortex as a result of stimulation by angiotensin II and others.^{1,2} Aldosterone has been demonstrated to bind to the mineralocorticoid receptor (MR) and to increase systemic blood pressure by regulating systemic electrolytes and volume balance in kidney, subsequently resulting in various human cardiovascular diseases.¹ However, aldosterone has also been demonstrated to directly exert its effects on cardiovascular systems via MR.^{1,3} For instance, aldosterone has been reported to induce expression of some genes involved in vascular fibrosis, calcification, and inflammation, which are all considered important in pathology of vascular injuries.¹ Aldosterone also induce mitogenesis of vascular smooth muscle cells (VSMCs), resulting in vascular structural remodeling under the presence of angiotensin II.^{4,5} However, aldosterone itself without the presence of angiotensin II is also considered to cause cardiovascular injuries.⁶ Vascular structural remodeling in small resistance arteries has been reported in patients with primary aldosteronism, where serum aldosterone levels were elevated but serum angiotensin II level is markedly down-regulated.⁷ In addition, aldosterone itself has been also demonstrated to stimulate proliferation of VSMCs.⁸ Therefore, aldosterone may directly induce some MR-responsive gene associated with regulation of the cell cycle in VSMCs, although inflammatory reaction and fibrosis are also very important features for aldosterone-induced vascular injuries and alterations.¹ Jaffe and Mendelsohn recently reported that some MR-mediated genes were associated with vascular injuries in VSMCs

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Table 1. Primers and Antibodies Used in This Study

Gene	Primer for PCR	Reference	Antibody	Reference
<i>MR</i>	Forward: CAGTCTCCAGTCCCAATAATGT Reverse: GCAGTGTAGCTGAAGGCATTGT		H10E4C9F (Alexis Corp.)	Lombes et al ¹³
<i>11β-HSD type 2</i>	Forward: ACGCAGGCCACAATGAAGTAG Reverse: GCAGCCAGGCTGGATGATGATG	Arcuri et al ¹²	HUH23	Krozowski et al ¹⁴
<i>MDM2 (TG)</i>	Forward: TGTAAGTGAACATTTCAGGTG Reverse: TTCCAATAGTCAGCTAAGGA	Ohtani et al ¹⁶	sc-965 (Santa Cruz)	Tan et al ¹⁹
<i>BIRC5</i>	Forward: TCCGGTTGCGCTTTTCCT Reverse: TCTTCTTATTGTTGGTTTCCTTTC	Williams et al ¹⁷		
<i>RPL13A</i>	Forward: CCTGGAGGAGAAGAGGAAAAG Reverse: TTGAGGACCTCTGTGTATTT			
<i>GAPDH</i>	Forward: TGAACGGGAAGCTCACTGG Reverse: TCCACCACCCTGTTGCTGTA			

It was determined that the target gene (TG) in this study was *MDM2* by microarray and real-time PCR analyses. The reason for this determination is described in detail in Results.

using microarray analysis.¹ Therefore, in this study, we first screened aldosterone-responsive genes involved in regulation of the cell cycle using microarray and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analyses as a confirmation of the findings in the cell line derived from MR-positive human VSMCs. We then used immunoblotting and immunofluorescence analysis to further evaluate the expression level of protein of the mineralocorticoid-responsive gene to further confirm the results of microarray analysis above. Eplerenone, a specific MR blocker, has been demonstrated to protect extrarenal tissues from various aldosterone-induced damage.^{1,3,9} Thus, in this study we also examined whether eplerenone may also inhibit an induction of this aldosterone-induced gene product in cultured human VSMCs. We then studied whether the gene product was involved in VSMC proliferation using small interfering RNA (siRNA) of the gene transfection. It then becomes important to examine relative abundance of the gene products in VSMCs of human cardiovascular system in correlation to serum aldosterone levels. So finally, we examined relative abundance of a gene product in VSMCs of human small resistance arteries obtained from patients of hypertension with primary aldosteronism and/or nonfunctioning adrenocortical tumors and normal or normotensive subjects using immunohistochemistry.

Materials and Methods

Cell Culture and Characterization

A cultured human VSMC cell line, ie, HASMC (derived from human abdominal aorta) was commercially obtained from Kurabo Corp. (Osaka, Japan). Characteristics of this cell line have been reported by Iseki et al.¹⁰ It was cultured in a 75-cm² flask with F12-K medium containing 5% fetal bovine serum (FBS) at 37°C in a 5% CO₂ atmosphere. We examined whether these cells expressed both MR and 11 β -hydroxysteroid dehydrogenases (11 β -HSD) type 2 using RT-PCR, immunoblotting, and immunocytochemistry, as reported previously.^{1,11} Primers and antibodies used in this study are summarized in Table 1.^{12–14}

Gene Chip Microarray Assay and Real-Time PCR Study

HASMC was cultured until a subconfluent state was obtained. The medium was then replaced with FBS-free and phenol red-free medium (modified Eagle's medium) (Sigma, St. Louis, MO) to arrest cell proliferation. After 24 hours, the medium was replaced again with phenol red-free and FBS-free medium in the presence of aldosterone (10 nmol/L) or vehicle (0.1% ethanol). After incubation for 8 hours, the cells were subsequently subjected to total RNA extraction for microarray analysis. Total RNA was prepared as previously described.¹¹ Microarray analysis was performed using a Human 1A Oligo Microarray (Agilent Technologies, Palo Alto, CA) with *in situ* synthesized 60-mer oligonucleotides representing 17,086 unique human genes. The procedures were described in detail in a previous report.¹⁵ In this study, the ratios represented the values up- or down-regulated by 10 nmol/L aldosterone treatment compared with control values. We independently repeated the same experiment twice, and the differences were calculated to further confirm aldosterone-related changes in gene expression obtained from microarray analysis. The ratios of genes increased by more than 2.0-fold by both replicates of 10 nmol/L aldosterone treatment were considered up-regulated via MR when compared with control values. When analyzing possible functions of these detected genes, we used the homepage of HUGO Human Gene Nomenclature Committee (<http://www.gene.ucl.ac.uk/nomenclature/>) for further examination. These results of microarray analyses were also confirmed by replicated quantitative RT/real-time PCR study. In this study, we regarded a gene that was also significantly up-regulated in RT/real-time PCR study as the target gene (TG) among the genes associated with mitogenesis and cell proliferation that were found to be significantly induced by aldosterone treatment in microarray analysis. Further RT/real-time PCR study was performed in the TG as follows. The VSMCs were further treated with aldosterone (100 pmol/L, 10 nmol/L), aldosterone (10 nmol/L) with eplerenone (100 nmol/L; Pfizer Inc., New York, NY), or vehicle. In addition, two additional flasks were treated with aldosterone (10

nmol/L) after pretreatment with inhibitors of RNA transcription, actinomycin D (ACD; 100 nmol/L; Sigma), or protein translation (Sigma), cycloheximide (CHX; 100 nmol/L; ICN Biomedicals Inc., Irvine, CA). After incubation for 8 hours, the cells were subsequently subjected to total RNA extraction for RT/real-time PCR analysis for target products mRNA expression. The detailed procedure of RT/real-time PCR analysis was previously described.¹¹ The mRNA levels for target product in each VSMC are summarized as a ratio of RPL13A and evaluated as a ratio (percentage) compared with that of each control cDNA. The information of primers used in this study is summarized in Table 1.^{16,17} We independently triplicated the same experiments for confirmation.

Immunoblotting Analysis

HASMC was cultured in the phenol red-free medium containing 5% FBS in the presence of aldosterone (10 nmol/L) with and/or without eplerenone (100 nmol/L) or in the presence of vehicle (0.1% ethanol). After incubation for 48 hours, the cells were subsequently subjected to nuclear extraction for immunoblotting analysis. An immunoblotting analysis was performed using 60 μ g of each protein. The detailed procedure was previously reported.^{11,18} In brief, protein samples were electrophoretically transferred to a polyvinylidene difluoride membrane, immunoblotted with antibodies against TG product, and visualized using chemiluminescence techniques.¹⁹ The relative amounts of TG protein levels for each band were standardized to the relative OD units as reported previously.¹¹ In addition, the relative amount of TG protein was evaluated as a ratio compared with control (percentage).¹⁸ We also independently triplicated the same experiments to confirm the findings.

Immunofluorescence

HASMC was cultured in the phenol red-free medium containing 5% FBS in the presence of aldosterone (10 nmol/L) with and/or without eplerenone (100 nmol/L) or in the presence of vehicle (0.1% ethanol). After incubation for 48 hours, the cells were subsequently fixed, stained, and visualized using TG antibody (Table 1), fluorescein isothiocyanate-conjugated secondary antibody, 4,6-diamidino-2-phenylindole for nuclear staining, and conventional fluorescence microscopy according to the manufacturer's instructions. We also independently triplicated the same experiments for confirmation.

siRNA Preparation, Transfection, and Cell Count Assay

siRNAs against TG were commercially obtained from Qiagen (Valencia, CA) and transfected to HASMC. HASMC was then seeded in a 6-well plate at an initial concentration of 100,000 cells/well with F-12K medium containing 5% FBS and cultured until a subconfluent status was achieved. The medium was then replaced

with phenol red-free and FBS-free medium to arrest the cell proliferation. After 24 hours, transfections of siRNA for endogenous gene targeting (0, 10, or 100 nmol/L) were performed with RNAiFect transfection reagent (Qiagen), respectively. After transfection, the cells were incubated in phenol red-free medium containing 5% FBS with aldosterone (10 nmol/L) with and/or without eplerenone (100 nmol/L) or in vehicle (0.1% ethanol). We measured the number of cells in each sample as described above after incubation for 48 and 72 hours, respectively, according to a previous report.¹⁹ We also examined the number of cells treated by aldosterone (10 nmol/L) with and/or without eplerenone (100 nmol/L), respectively. To evaluate efficiency of transfection to the cells, we examined relative TG mRNAs levels in these cells at 24 hours after transfection of the specific siRNAs. As a positive control, transfections of siRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 0, 10, or 100 nmol/L; Amersham Biosciences, Piscataway, NJ) were also performed, and relative GAPDH mRNAs were evaluated in the cells. The mRNA levels in each VSMC are summarized as a ratio of RPL13A and were normalized as the ratio at no treatment (0 nmol/L), respectively.

Human Adrenal Specimens

Thirty-six cases of adrenocortical tumors (24 primary aldosteronism associated with hypertension and 12 non-functioning adenomas associated with no clinical hormonal abnormalities, including normal aldosterone level but with hypertension) were retrieved from surgical pathology files of Tohoku University Hospital. In addition, 12 specimens of histopathologically normal adrenal glands were obtained from autopsy files of individuals without histories of hypertension (from Tohoku University Hospital, Sendai, Japan). These specimens were fixed in 10% formalin for 24 to 48 hours at room temperature and embedded in paraffin wax. The research protocols for this study were approved by the ethics committee at Tohoku University School of Medicine (2004-355).

Immunohistochemistry

Immunohistochemical analysis was performed using the specific antibody against TG protein (Table 1) by streptavidin-biotin amplification method using a Histofine kit (Nichirei, Tokyo, Japan), as previously described in detail.^{11,19,20} After a thorough review of immunostained tissue sections, relative immunoreactivity for TG protein in VSMCs of at least 25 resistant small arteries (100 to 300 μ m in diameter) adjacent to adrenal tumors or normal glands per case were evaluated by labeling index (LI), a quantitative value that evaluates the numbers of cells positive for TG immunoreactivity in VSMCs.¹¹ The LIs were independently and blindly evaluated by three of the authors (Y.N., T.S., and H.S.) to obtain immunohistochemical data objectively, and the mean of the three values was used for analysis.

Figure 1. A: Representative RT-PCR analysis of total RNA from cultured human VSMCs (HASMC). RNA was amplified in the presence of oligonucleotide primers specific for MR (**top panel**) and 11β-HSD type 2 (**bottom panel**). Extracts from human kidney were used as a positive control (P). **B:** Representative immunoblotting studies demonstrating MR and 11β-HSD type 2 proteins in HASMC. Extracts from human kidney were used as a positive control (P). **C and D:** Immunocytochemical analysis demonstrated that immunopositive cells for MR appear brown as a result of diaminobenzidine colorimetric reaction in HASMC without ligand stimulation (**C**) or treated with 10 nmol/L aldosterone (**D**) (Original magnification, ×400).

Statistical Analysis

Values for all results are shown as mean ± SD. We used one-way analysis of variance followed by the Bonferroni test for comparisons between two different groups. A *P* value less than 0.05 was considered significant in this study.

Results

MR Expression in HASMC

RT-PCR study confirmed that both MR and 11β-HSD type 2 mRNAs were detected in HASMC (Figure 1A). Negative

Table 2. Aldosterone-Induced Genes Demonstrated by Microarray and Quantitative RT/Real-Time PCR (qRT-PCR) Analyses

Gene	Molecular function	Microarray fold change	qRT-PCR	
			Fold change	<i>P</i> value
<i>MDM2</i>	Cell growth/ inhibitor of apoptosis	2.2	2.3 ± 0.4	<0.05
<i>BIRC5</i>	Inhibitor of apoptosis	2.0	1.4 ± 0.5	
<i>LCP1</i>	Immune response/ inflammation	3.2	Not examined	Not significant
<i>IGSF4B</i>	Immune response/ inflammation	3.0	Not examined	
<i>CMRF-35H</i>	Immune response/ inflammation	2.8	Not examined	
<i>IL1R2</i>	Immune response/ inflammation	2.8	Not examined	
<i>SLC13A5</i>	Citrate transporter	2.8	Not examined	
<i>SLC22A8</i>	Organic anion transporter	2.3	Not examined	

Ratios represent mean fold activation of gene expression in VSMCs treated by aldosterone for 8 hours compared with vehicle control. Results of qRT-PCR are shown as mean ± SD.

Figure 2. Results of RT/real-time PCR analysis for *MDM2* in HASMC among cells treated with vehicle (0.1% ethanol) (control), aldosterone alone (100 pmol/L, 10 nmol/L), aldosterone (10 nmol/L) with eplerenone (100 nmol/L), aldosterone (10 nmol/L) with CHX, and aldosterone (10 nmol/L) with ACD (100 nmol/L), respectively, after 8 hours. Data are shown as mean $\pm$ SD (* P < 0.05).

controls demonstrated no discernible bands (data not shown). MR and 11 β -HSD type 2 proteins were also detected in HASMC (Figure 1B). Cellular localization of MR protein in the cells was also studied by immunocytochemical analysis. It was demonstrated that MR protein expression was detectable in both the nucleus and the cytoplasm without ligand stimulation (Figure 1C). After exposure to aldosterone, MR was solely found in the

nucleus of the cells (Figure 1D). These findings were similar to results of previous study.¹

GeneChip Microarray Assay and RT/Real-Time PCR Study

Table 2 summarized the genes associated with expression ratios of above 2.0 after 8 hours of duplicated 10 nmol/L aldosterone treatment in MR-positive HASMC. These genes were related to mitogenesis/cell growth, inflammation, and transporter of various substances. Among these genes, *MDM2* and *BIRC5* were known to be involved in cell proliferation and anti-apoptosis.^{16,17,21} In addition, among these two genes, only *MDM2* was significantly promoted by aldosterone compared with control in a RT/real-time PCR study (Table 2). Therefore we further examined the features of *MDM2* as an aldosterone-responsive gene in HASMC and whether *MDM2* was associated with aldosterone-induced promotion of MR-positive VSMC proliferation using further quantitative RT/real-time PCR, immunoblotting analysis, immunofluorescence analysis, and siRNA transfection assay described above. Results of RT/real-time PCR study demonstrated that aldosterone with CHX also significantly increased *MDM2* expression in HASMC compared with controls (P < 0.05) (Figure 2). However, aldosterone with eplerenone and aldosterone with ACD did not increase expression of these mRNAs (Figure 2). Results of both

Figure 3. A: Representative immunoblotting studies demonstrating *MDM2* proteins in HASMC among cells treated with vehicle (0.1% ethanol) (control), aldosterone alone (10 nmol/L), and aldosterone (10 nmol/L) with eplerenone (100 nmol/L), respectively, after 48 hours. **B:** Relative levels of *MDM2* protein expression in HASMC among cells treated with vehicle (0.1% ethanol) (control), aldosterone alone (10 nmol/L), and aldosterone (10 nmol/L) with eplerenone (100 nmol/L), respectively, after 48 hours. Data are shown as mean $\pm$ SD (* P < 0.05).

Figure 4. Representative immunofluorescence studies demonstrating *MDM2* proteins in HASMC among cells treated with vehicle (0.1% ethanol) (control) (A), aldosterone alone (10 nmol/L) (B), and aldosterone (10 nmol/L) with eplerenone (100 nmol/L) (C), respectively, after 48 hours. *MDM2* protein expression (green) was remarkably abundant in the nucleus of cells treated by aldosterone using fluorescein isothiocyanate (FITC)-conjugated secondary antibody (B), and its expression was blocked by eplerenone (C). 4,6-Diamidino-2-phenylindole (DAPI) was used for nuclear staining (blue).

microarray and quantitative RT/real-time PCR analyses also demonstrated that *MDM2* is one of the early genes induced by aldosterone via MR in HASMC.

MDM2 Protein Expression Study in Immunoblotting and Immunofluorescence

In the immunoblotting study, aldosterone significantly increased *MDM2* protein expression levels in HASMC compared with controls after 48 hours of incubation ($P < 0.05$) (Figure 3). However, aldosterone with eplerenone did not promote expression of these proteins (Figure 3). In the immunofluorescence study, aldosterone extensively increased *MDM2* protein located in the nucleus of HASMC compared with controls after 48 hours of incubation (Figure 4, A and B). However, aldosterone with eplerenone did not induce *MDM2* protein expression (Figure 4C). Results of these analyses also demonstrated that *MDM2* protein was extensively induced by aldosterone via MR and inhibited by a specific MR blocker, eplerenone.

MDM2 siRNA Transfections and Cell Proliferation Assay

A down-regulation of *MDM2* and/or GAPDH mRNA levels was dose dependently confirmed in the cells by transfection of *MDM2* and/or GAPDH siRNAs (10 and 100 nmol/L) using RT/real-time PCR, respectively (Figure 5A). Under the absence of transfection of siRNA (0 nmol/L), aldosterone significantly promoted proliferation of HASMC compared with controls (approximately 1.3- to 1.4-fold after 48 and/or 72 hours) (Figure 5, B and C). However, under the transfection of *MDM2* siRNA (10 and 100 nmol/L), aldosterone did not promote the cell proliferation of HASMC compared with control (Figure 5, B and C). On the other hand, under eplerenone, aldosterone did not significantly promote cell proliferation of HASMC induced by aldosterone (Figure 5, B and C).

MDM2 Immunoreactivity in Human Resistance Artery

Figure 6 demonstrates representative examples of human small resistance arteries adjacent to normal adrenal gland or adrenal tumor specimen obtained by surgery. Relative immunoreactivities of *MDM2* were significantly higher in VSMCs of human small resistance arteries adjacent to adrenocortical adenoma of primary aldosteronism associated with hypertension than normal adrenal gland in normotensive patients and/or nonfunctioning adrenocortical adenoma with and/or without hypertension ($P < 0.05$).

Discussion

In our present study, results of both microarray and quantitative RT-PCR analyses and immunoblotting and immunofluorescence studies all suggest that *MDM2* is one of the genes induced by aldosterone via MR in cultured human VSMCs. In addition, results of siRNA analysis demonstrated that *MDM2* is possibly involved in VSMC proliferation through MR by aldosterone. Furthermore, results of immunohistochemical study in human arteries revealed that *MDM2* was markedly expressed in VSMCs of small arteries in the patients with aldosterone-producing adrenocortical adenoma compared with other groups including the hypertensive patients with nonfunctioning adrenocortical adenomas, ie, no clinical aldosterone abnormalities. These findings above indicate that an expression of *MDM2* is strongly induced by aldosterone itself via MR in VSMCs both *in vitro* and *in vivo*, and *MDM2* may be possibly involved in aldosterone-induced vascular structural remodeling of human resistance arteries, which may result in persistent hypertension even after resection of aldosterone-producing adrenocortical adenoma.²²

VSMCs used in this study were also associated with 11β -HSD type 2 expression. It is known that both aldosterone and cortisol bind to human MR with equal affinity.^{1,23} Plasma glucocorticoid concentrations are 100- to

Figure 5. **A:** Expression of *MDM2*, *GAPDH*, and *RPL13A* mRNAs at 24 hours after the transfection of siRNA (0, 10, or 100 nmol/L) against *MDM2* and/or *GAPDH* in HASMC cells detected by real-time PCR, respectively. *RPL13A* expression was monitored as the control. The ratio of *MDM2* to *RPL13A* and/or *GAPDH* to *RPL13A* was calculated, and values were normalized as the ratio at no treatment (0 nmol/L), respectively. **B:** The levels of cell numbers in HASMC at 48 hours after treatment with vehicle (0.1% ethanol) (control) or aldosterone (10 nmol/L) after transfection of *MDM2* siRNA (0, 10, or 100 nmol/L) or under the presence of eplerenone (100 nmol/L). Data are presented as mean $\pm$ SD (* P < 0.05). **C:** The levels of cell numbers in HASMC at 72 hours after treatment with vehicle (0.1% ethanol) (control) or aldosterone (10 nmol/L) after transfection of *MDM2* siRNA (0, 10, or 100 nmol/L) or under the presence of eplerenone (100 nmol/L). Data are represented as mean $\pm$ SD (* P < 0.05). N.S., not significant.

1000-fold higher than those of aldosterone, but aldosterone-responsive tissues express the cortisol-inactivating enzyme 11 β -HSD type 2, which converts cortisol to its 11-keto derivatives that have a lower affinity for MR.^{1,24} 11 β -HSD type 2 is abundant in the kidney, making renal cells highly responsive to aldosterone.^{1,25,26} It is known that VSMCs also express a functional 11 β -HSD type 2 capable of inactivating cortisol, as in other aldosterone target tissues.¹ In addition, the presence of both MR and 11 β -HSD type 2 has been reported in human aorta.^{1,27} Therefore, HASMC is considered an appropriate model for *in vitro* examination of direct effects of aldosterone in human cardiovascular system.

Results of our present study did demonstrate that aldosterone itself may possibly exert cell proliferative effects on VSMCs *in vitro* through MR by aldosterone to some extent (approximately 1.3- to 1.4-fold compared with control). Angiotensin II is well known to be required for mitogenic effects of aldosterone in a lower dose in rat VSMCs, and the pathway via extracellular signal-regulated kinase activation has been considered to be involved in aldosterone-induced VSMC mitogenesis under the presence of angiotensin II.^{4,5} In addition, Ishizawa et al also reported that aldosterone itself possibly induced proliferation of VSMCs (less than 1.5-fold compared with control), which suggest that proliferative effects of aldosterone alone on

VSMCs are not necessarily marked as demonstrated in our present study.⁸ However, higher plasma aldosterone levels have been demonstrated to be associated with the status of in-stent restenosed coronary arteries in patients with angina, which suggest that aldosterone is involved in VSMC proliferation and vascular remodeling in these patients.²⁸ In addition, inflammation, fibrosis, and calcification have been considered to play important roles in vascular injuries caused by aldosterone.¹ Therefore, these findings all suggest that VSMC proliferation induced by aldosterone itself is one of the important factors on vascular remodeling and is possibly accelerated by other factors including inflammation and angiotensin II.

MDM2 is a nuclear protein that forms a complex with p53.²¹ *MDM2* is known to regulate the biological activity of p53 by preventing p53-mediated apoptosis or reversing p53-induced G1 block of the cell cycle and thus promoting the entry of cells into S phases through formation of these complexes.^{21,29,30} In addition, *MDM2* has been also considered to be involved in promoting the entry of cells from G2 into M phases by reversing p53-mediated G2 block of the cell cycle.^{21,29,31} *MDM2* is also known to interact both physically and functionally with the RB protein, which is also involved in VSMC growth.^{21,32-34} *MDM2* was also reported to be regulated by the Ras-driven Raf/MEK/

Figure 6. A–C: Immunohistochemistry for *MDM2* in VSMCs of small resistance arteries adjacent to adrenal tumors or normal adrenal glands. Immunoreactivity for *MDM2* in VSMCs of small resistance arteries adjacent to adrenal tumor of primary aldosteronism (A), nonfunctioning adrenal tumor (B), and non-neoplastic adrenal gland (C). Immunoreactivity appears brown as a result of diaminobenzidine colorimetric reaction. Original magnification, $\times 400$. D: The relative immunoreactivity was evaluated by the LI in each group (0 to 100). Data are represented as mean $\pm$ SD (* $P < 0.05$). PA, aldosterone-producing adrenocortical adenoma; NF, nonfunctioning tumor; normal, non-neoplastic adrenal gland.

MAP kinase pathway, in a p53-independent manner.³⁵ Several investigators reported that *MDM2* may also play an important role in atherogenesis in human atherosclerotic plaques.^{21,32,33,36} Results of our present study suggest that *MDM2* may be involved in regulation of a cell cycle and cell proliferation by aldosterone itself in human VSMCs, which is consistent with these previous studies.^{21,29–36}

In our present study, the patients associated with both increased plasma aldosterone levels and hypertension demonstrated significantly more *MDM2* immunoreactivity in VSMCs of small arteries than those with hypertension but normal plasma aldosterone levels. Therefore, *MDM2* is considered to be at least induced by relatively high levels of plasma aldosterone but not necessarily by effects of hypertension alone and to be involved in vascular remodeling frequently detected in the patients with primary aldosteronism.⁷ However, it is also true that VSMC proliferation may occur in response to hypertension itself.³⁷ Therefore, the *MDM2* pathway demonstrated in this study is certainly not the only factor involved in vascular structural remodeling associated with elevated lev-

els of plasma aldosterone. Further investigations of direct aldosterone effects toward human cardiovascular system are required for clarification.

The MR-mediated effects of aldosterone are considered genomic ones through binding of aldosterone to the intracellular MR and the translocation of the steroid-MR complex to the nucleus, where it acts as a transcriptional regulator, resulting in its biological effects after several hours.³⁸ In addition, quantitative RT-PCR analysis in our present study demonstrated that ACD suppressed aldosterone-induced *MDM2* mRNA expression, but CHX did not inhibit its expression. Therefore, these findings all demonstrated that *MDM2* is considered one of the first established responsive genes in MR-positive VSMCs. However, it awaits further investigations to clarify the exact mechanisms of *MDM2* induction pathway.

Results of real-time PCR, immunoblotting, immunofluorescence, and cell proliferation studies also demonstrated that eplerenone may inhibit *MDM2* induction by aldosterone in MR-positive VSMCs. Eplerenone may also confer cardiovascular protective effects on aldo-

sterone-induced VSMC proliferation and vascular remodeling through this pathway above. Aldosterone antagonists have been demonstrated to inhibit both of these vascular injuries by aldosterone in some clinical and experimental studies in cardiovascular system.^{1,3,9} Eplerenone is a selective aldosterone blocker that provides clinical efficacy in the treatment of hypertension and heart failure compared with spironolactone, a relatively nonselective aldosterone blocker in view of its side-effect profile.⁹ These findings, including results of our present study, all demonstrated that eplerenone may prevent vascular damages through this *MDM2* pathway at least in the patients with primary aldosteronism. In conclusion, *MDM2* is considered one of the mineralocorticoid-responsive genes involving MR-mediated VSMC proliferation and may play important roles in aldosterone-related vascular structural alterations of human cardiovascular system.

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