transfected using FuGene6 (Roche Diagnostics K.K., Tokyo, Japan), following the manufacturer's instructions, and the following day treated with either ethanol or 10 nM E2. Typically, cells were transfected with 0.3 μg luciferase reporter plasmid, 50 ng pERαcDNA3.1 (ERα expression plasmid) or 50 ng pRL-TK reporter plasmid (cDNA encoding *Renilla* luciferase downstream of the thymidine kinase promoter) (Promega, Tokyo, Japan). Luciferase values were normalized to the *Renilla* luciferase activity.

Acknowledgements

This work was supported in part by a Core Research for Evolutional Science research grant from the Japan Science and Technology Cooperation, a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a Health Sciences Research Grant from the Ministry of Health, Labour and Welfare of Japan, a research grant from the Ministry of Environment, Japan.

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Received: 10 April 2006
Accepted: 17 May 2006

The estrogen-responsive adrenomedullin and receptor-modifying protein 3 gene identified by DNA microarray analysis are directly regulated by estrogen receptor

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Abstract

Recent studies have revealed that hundreds of genes in the uterus are activated by estrogen. Their expression profiles differ over time and doses and it is not clear whether all these genes are directly regulated by estrogen via the estrogen receptor. To select the genes that may be regulated by estrogen, we treated mice with several doses of estrogen and searched for those genes whose dose–response expression pattern mirrored the uterine growth pattern. Among those genes, we found that the dose-dependent expression of the adrenomedullin (ADM) gene correlated well with the uterotrophic effect of estrogen. ADM expression is induced early after estrogen administration and is restricted to the endometrial stroma. The spatiotemporal gene expression pattern of ADM was similar to that of receptor-modifying protein 3 (RAMP3). RAMP3 is known to modify calcitonin gene-related receptor (CRLR) so that it can then serve as an ADM receptor. Chromatin immunoprecipitation assays indicated that the estrogen receptor binds directly to the ADM promoter region and RAMP3 intron after estrogen administration. It was also shown that neither the ADM nor RAMP3 gene could be activated in estrogen receptor-α-null mouse. Although uterine ADM expression has been reported to occur in the myometrium, our observations indicate that estrogen-induced ADM is also expressed in the uterine stroma and that such variable, spatiotemporally regulated ADM expression contributes to a wider range of biological effects than previously expected.

Journal of Molecular Endocrinology (2006) **36**, 81–89

Introduction

The uterus is a major target organ of estrogen and undergoes drastic changes after estrogen administration. Early uterine responses to estrogen include many physiological biochemical changes such as hyperemia, calcium influx, histamine release, eosinophil infiltration, cAMP level changes, enhanced glucose oxidation, and increased RNA and protein synthesis. After DNA synthesis and mitosis, later stage effects include cellular hypertrophy and hyperplasia and the result is the growth of the uterus. This uterotrophic effect of estrogen is a demonstrable phenotype that can be used to estimate the potency of estrogens. Notably, not all the physiological and biochemical changes associated with estrogen treatment are necessary for the uterotrophic effect. For example, while cAMP is elevated after estrogen administration (Szego & Davis 1967), uterine growth is independent of cAMP (Zor *et al.* 1973). This is also true for ornithine decarboxylation and prostaglandins. Thus, it remains unclear which processes are essential for the effects of estrogen in target tissues.

In the level of gene expression, it is also not clear whether estrogen-response genes are directly regulated by estrogen receptor (ER) or not. Recently, the effect of estrogen on the uterus has been studied at the level of gene expression by DNA microarray analysis (Watanabe *et al.* 2002). As a result, hundreds of genes have been listed as being estrogen-responsive and the temporal changes in their expression after estrogen exposure have been analyzed (Watanabe *et al.* 2003). This has vastly aided our understanding of the effect of estrogen on the uterus at the genetic level. However, this new methodology, like the classical methodology, cannot determine whether a particular gene-expression change is the direct effect of estrogen or not.

In our previous study we found that not all estrogen-responsive genes are uniformly activated by estrogen and that their estrogen-dose-dependent, gene-expression patterns are not necessarily identical with the uterotrophic effect of estrogen (Watanabe *et al.* 2003). For example, while the uterotrophic effect of estrogen is directly proportional to the estrogen doses used, some

Journal of Molecular Endocrinology (2006) **36**, 81–89
0952–5041/06/036–081 *Printed in Great Britain*

DOI: 10.1677/jme.1.01825
Online version via http://www.endocrinology-journals.org

genes are activated by a low dose of estrogen but this activation is saturated or diminished by higher doses of estrogen. These various estrogen-response patterns are generally attributed to the differences of promoter context but the contribution of ER is not clear. In this study, we selected the adrenomedullin (ADM) and receptor-modifying protein 3 (RAMP3) genes, whose expression patterns correlate closely with the uterotrophic effect, and studied the direct contribution of ER to their expression.

Experimental procedures

Animals

Female C57BL/6J mice and ERα- and -β-null mice (Dupont *et al.* 2000) were housed under a 12 h:12 h light/darkness cycle. To assess the effect of estrogen on uterine gene expression, mice were ovariectomized at 8 weeks of age and 2 weeks later injected intraperitoneally with estrogen (or sesame oil as a vehicle control; Nakarai Tesque, Kyoto, Japan). The whole uterus ($n=4$) were collected 6 h later. The estrogen used was 17β-estradiol (Sigma-Aldrich Japan, Tokyo, Japan) and it was injected at 0·05, 0·5, 5 or 50 μg/kg body weight (b.w.). To assess the effects of the different estrogen doses on uterine growth, mice were ovariectomized and 2 weeks later injected intraperitoneally every 24 h for 7 days with the different 17β-estradiol doses or sesame oil. The whole uterus ($n=5$) were then collected and weighed. All animal experiments were approved by the institutional animal care committee.

Preparation of labeled cRNA and microarray analysis

Total uterine RNA was extracted using TRIzol reagent (Invitrogen, Tokyo, Japan) and cRNA probes were prepared from the purified RNA by using an Affymetrix cRNA probe kit according to the manufacturer's protocol (Affymetrix Japan, Tokyo, Japan). All preparations met the recommended criteria of Affymetrix for use on their expression arrays. The amplified cRNA was hybridized to high-density oligonucleotide arrays (Mouse U74 A; Affymetrix Japan), and the scanned data were analyzed with GeneChip software (Affymetrix Japan) and processed as described previously (Watanabe *et al.* 2002). To confirm the estrogen-related changes in gene expression revealed by the DNA microarray analysis, we independently repeated the same experiment at least twice. The expression data were analyzed with GeneSpring software (Agilent Tech. Japan, Tokyo, Japan).

Quantitative real-time PCR

cDNA was synthesized from total RNA purified as described above by using Superscript II reverse transcriptase (Invitrogen) with random primers at 42 °C for 60 min. Quantitative PCRs were performed by using the PE Prism 7000 sequence detector (Applied Biosystems, Tokyo, Japan), SYBR-Green PCR core reagents (Applied Biosystems) and the appropriate primers according to the manufacturer's instructions. The primers were chosen to amplify short PCR products (<100 bp), and their sequences were as follows: RAMP1 (NM_016894), 5′-TTTCATTGCGCTCCCCATT-3′ and 5′-CCAGACCACCAGTGCAGTCAT-3′; RAMP2 (NM_019444), 5′-AGTTGCATGGACTCTGTCAAG GA-3′ and 5′-TGCCTGCTAATCAAAGTCCAGTT-3′; RAMP3 (NM_019511), 5′-CCGGATGAAGTACT CATCCCA-3′ and 5′-CCACCAGGCCAGCCATAG-3′; ADM (U77630) 5′-ATAAGCCTCATTACTACTT GAACT-3′ and 5′-TTGCACGTTCCTCGCTAGGT-3′, and CRLR (calcitonin gene-related receptor; NM_018782) 5′-CACTCTGATGCTCTCCGCAGT-3′ and 5′-GGCTGTACCCTTGCATGTCAC-3′. Gene-expression levels were normalized to the expression levels of NM_012053 (ribosome L8, Rpl8), whose primer sequences were 5′-ACAGAGCCGTTGTTGGTGT TG-3′ and 5′-CAGCAGTTCCTCTTTGCCTTGT-3′. Gel electrophoresis and melting-curve analyses were performed to confirm correct amplicon size and the absence of nonspecific bands.

In situ hybridization

In situ hybridization was performed using PCR fragments containing a portion of the ADM gene (nucleotides 58–586; U77630), the RAMP2 gene (nucleotides 160–706; NM_019444) or the RAMP3 gene (nucleotides 681–1059; NM_019511). [α-^{35}S]UTP (Amersham Biosciences, Tokyo, Japan)-labeled antisense and sense RNAs were obtained by using T7 and T3 RNA polymerases and an *in vitro* transcription kit (Stratagene, Funakoshi, Tokyo, Japan). After DNase digestion, the probes were fragmented by alkali hydrolysis. The tissues were embedded in compound (Sakura Fintechnical Co., Ltd., Tokyo, Japan) and sectioned (10 μm) using a cryostat. Sections were fixed on slides with 4% paraformaldehyde for 10 min and rinsed in PBS. The slides were then acetylated in 0·1 M triethanolamine with a 1/400 vol. acetic anhydride, rinsed again in PBS, and dehydrated in graded ethanol. After air drying, the hybridization mixture was added. Hybridization was performed at 50 °C overnight with 5×10^7 d.p.m./ml probe in 100 μl hybridization solution under coverslips. After the hybridization, the slides were washed and incubated in RNase A solution (20 μg/ml) at 37 °C for 30 min, and then dipped in NTB-2 nuclear track emulsion (Kodak, Rochester, NY, USA). After 14 days exposure, the slides were developed and counterstained with hematoxylin. The sections were evaluated and photographed under dark-field illumination using a Zeiss microscope. The dark-field (changed to red) and bright-field images were merged.

Chromatin immunoprecipitation

Mouse uteri were fixed with 1% formaldehyde and homogenized using physcotron (NS-310E; Microtec, Chiba, Japan) in PBS containing 0·125 M glycine. The samples were then centrifuged at 700 ***g*** for 5 min at 4 °C and the pellets were incubated with lysis buffer (10 mM Tris/HCl, pH 8·0, 10 mM EDTA, 0·5 mM EGTA and 0·25% Triton X-100) for 10 min. The samples were collected by microcentrifugation, suspended in sonication buffer (10 mM Tris/HCl, pH 8·0, 100 mM NaCl, 1 mM EDTA and 0·5 mM EGTA), and sonicated with a Bioruptor sonicator (Cosmo Bio, Tokyo, Japan) to an average length of approximately 500 bp. The samples were precleared by treatment with Protein G–Sepharose for 1 h at 4 °C and then incubated with 10 μg anti-ERα polyclonal rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-acetylated histone H3 antibody (Cell Signaling, Beverly, MA, USA) overnight at 4 °C. After precipitation by the addition of Protein G–Sepharose the samples were washed five times with RIPA buffer (10 mM Tris/HCl, pH 8·0, 140 mM NaCl, 1 mM EDTA, 0·5 mM EGTA, 1% Triton X-100, 0·1% SDS and 0·1% sodium deoxycholate). The precipitated samples were recovered by incubation with elution buffer (0·1 M sodium bicarbonate and 1% SDS). Crosslinks were reversed by incubation at 65 °C for 6 h followed by incubation with proteinase K at 45 °C for 4 h. Thereafter, the samples were extracted with phenol/chloroform and the DNA fragments were precipitated with a 1/10 vol. of NaCl and 2·5 vol. of ethanol. Generally, 1/30 of the precipitated DNA was used for PCR amplification. PCR amplification was performed in the presence of 0·1 nmol primers, 0·2 mM each nucleotide (dATP, dCTP, dGTP and dTTP), 1 × PCR buffer and 1 U AmpliTaq Gold (PerkinElmer Japan, Tokyo, Japan) in 20 μl of reaction buffer. After 35 cycles of amplification the amplified DNA was analyzed by agarose electrophoresis. As a negative control the same experiments were performed with IgG: no amplified DNA was obtained. The primer sequences used to amplify the putative estrogen-response element (ERE) of the ADM gene were (−796) 5′-ATCCTCA CGTTTATGATGGA-3′ (−777) and (−565) 5′-CGG ATTTCGTAATAAGGGCA-3′ (−584). The primer sequences used to amplify the putative ERE of the RAMP3 gene were (13 531 bp from the 5′ end of the first intron) 5′-AGAGTGTACGTGTGGACAGG-3′ (13 550 bp) and (13 735 bp from the 5′ end of the first intron) 5′-CTGTGACAGCAGGAGGACAG-3′ (13 716).

Results

The ADM gene expression pattern correlates well with the uterotrophic effect of estrogen

To select genes that may be involved in the estrogen-induced uterine response, we injected ovariectomized mice with four different doses of estradiol and harvested the uteri 6 h later. The total uterine RNAs were then subjected to DNA microarray analysis, which determined the expression levels of 10 000 genes. The average gene-expression levels were compared with the estrogen dose response of uterine growth (determined by daily injecting ovariectomized mice for a week with the four different estrogen doses and then weighing the whole uteri; Fig. 1A). The genes whose expression levels correlated well with the uterotrophic effect of estrogen were selected.

Of the 10 000 genes examined, 338 had a correlation coefficient of greater than 0·95. The average expression levels of these genes, as determined by the microarray analysis, are shown in Fig. 1B. The 338 selected genes included the ADM gene (Fig. 1C). In addition, the RAMP3 gene displays a similar estrogen-induced expression pattern, as shown by Fig. 1D. This is of interest because RAMP3 modifies the calcitonin gene-related receptor (CRLR), after which CRLR can serve as a receptor for ADM (McLatchie *et al.* 1998). However, CRLR gene expression was unaffected by estrogen treatment (Fig. 1D). The good correlation between ADM and RAMP3 gene expression prompted us to examine the gene-expression regulation of ADM and RAMP3 by estrogen.

ADM and RAMP3 gene expression are activated early after estrogen administration

To examine the temporal changes in ADM gene expression by estrogen exposure, we injected ovariectomized mice with 5 μg/kg b.w. estradiol, harvested the uteri 1, 2, 4 or 6 h later, and subjected the whole uterine RNA to quantitative PCR to evaluate ADM expression. The experiment was performed three times. While ADM gene expression in the uteri varied quite widely 2–4 h after estrogen administration, it was elevated 1 h after estrogen administration and continued to increase thereafter until around 4 h after estrogen administration, after which ADM expression started to decrease (Fig. 2A). Thus ADM gene expression was induced early after estrogen stimulation. RAMP3 gene expression was also induced immediately after estrogen administration and continued until 4 h after estrogen administration (Fig. 2B).

There is also another modifying protein that confers ADM receptor function onto CRLR, namely RAMP2 (McLatchie *et al.* 1998). However, we found RAMP2 expression was not effectively activated by estrogen (Fig. 2C). Moreover, RAMP1, a third CRLR-modifying protein that modifies CRLR to act as a receptor for calcitonin-gene-related peptide (CGRP; McLatchie *et al.* 1998), was substantially repressed by estrogen (Fig. 2C). Furthermore, CGRP, which binds to receptors bearing RAMP3 or RAMP1 (McLatchie *et al.* 1998), was

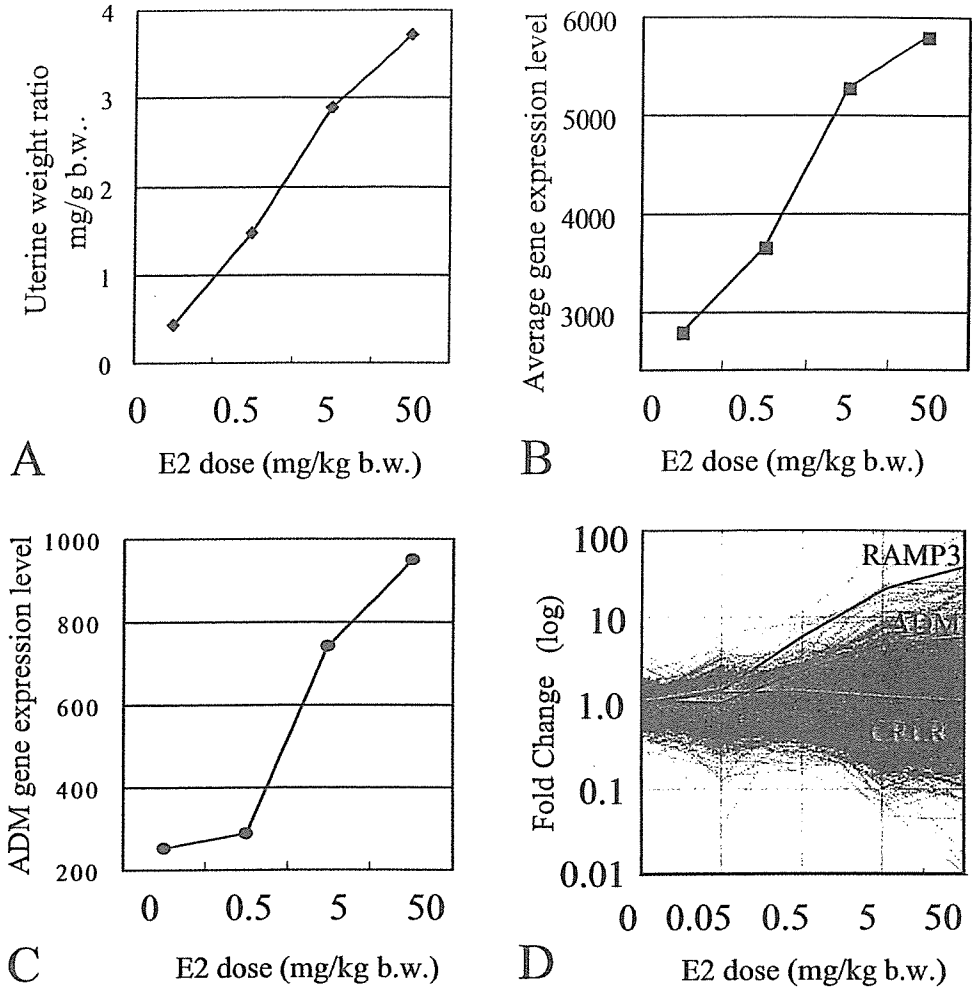

Figure 1 Correlation between estrogen dose-dependent uterine ADM gene expression and the uterotrophic effect of estrogen. (A) Uterotrophic effect of estrogen. Estrogen dose-dependent uterine growth is indicated by a ratio to body weight. (B) Average gene-expression levels of the selected genes; 338 genes whose expression correlated well with the uterotrophic effect were selected and the average gene-expression levels of the 338 genes at the different estrogen doses (calculated from the fluorescent signal intensity of each gene in the DNA microarray analysis) are indicated. (C) Estrogen-dependent gene activation of ADM gene. The gene-expression level of ADM (one of the 338 genes) is indicated. The *x* axis indicates the estradiol dose administered while the *y* axis indicated the gene-expression levels. (D) Estrogen dose-dependent changes in ADM, RAMP3 and CRLR gene expression (solid lines) relative to their expression levels at 0 μg/kg b.w. estradiol (fold expression). Gene expression of all the 10 000 genes examined is shown in gray. While ADM and RAMP3 gene expression were activated by estrogen, CRLR gene expression was not changed. The *x* axis indicates the estradiol dose administered while the *y* axis indicates fold changes in gene expression on a log scale. Note that because of the normalization algorithm, the gray gene expression is not exactly 1·0 but spread around 1·0 at the 0 mg/kg b.w. dose. E2, 17β-estradiol.

expressed in the absence of estrogen at much lower levels than ADM and was unaltered by estrogen administration (data not shown).

To examine how the expression levels of RAMP3 relate to CRLR expression upon estrogen exposure, the ratio of RAMP3 mRNA to CRLR mRNA was calculated from data obtained by quantitative PCR. As indicated in Fig. 1D, CRLR expression is unaltered by estrogen. Although RAMP3 mRNA levels were one-tenth of those of CRLR before estrogen administration, estrogen induced RAMP3 gene transcription, with the result that RAMP3 and CRLR were expressed at equivalent levels 2 h after estrogen administration, shown by mRNA levels. Although protein-level confirmation is

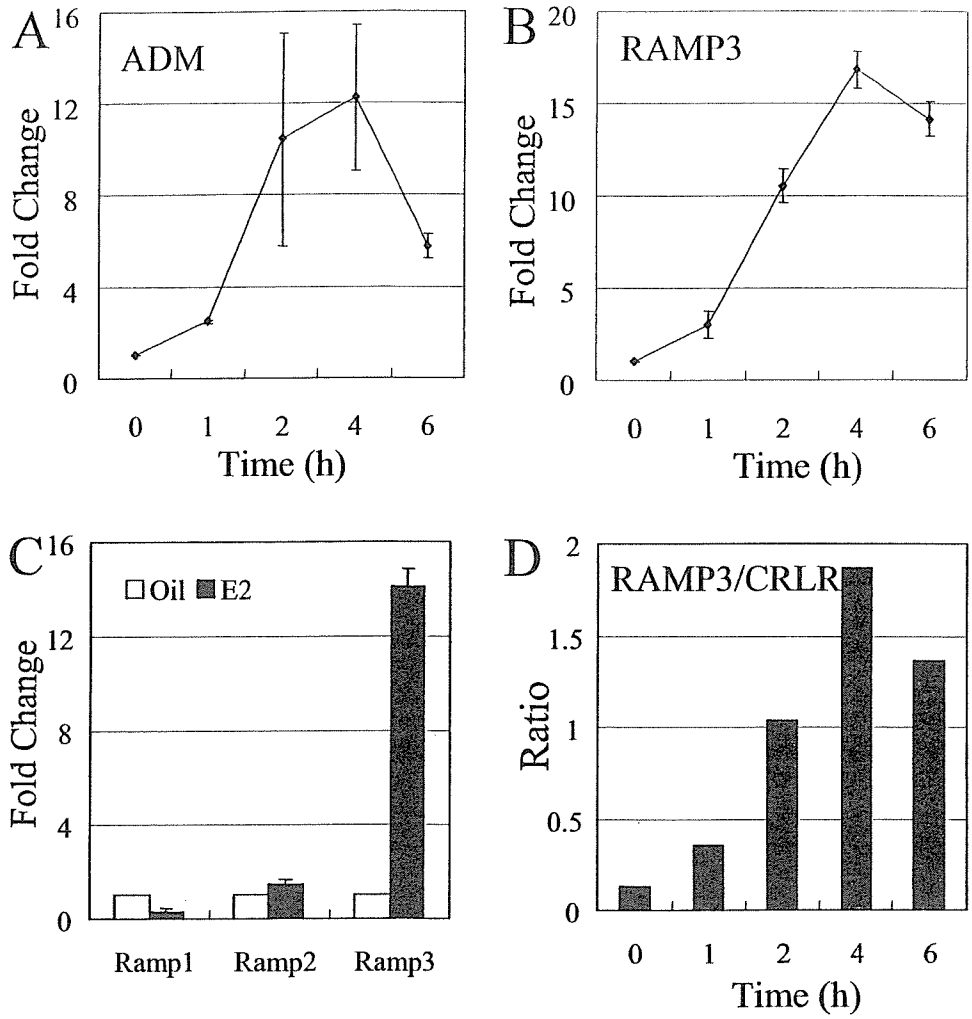

Figure 2 Temporal changes in ADM and RAMP3 gene expression. Ovariectomized mice were injected with 5 μg/kg b.w. estradiol and total uterine RNA was isolated 1, 2, 4 and 6 h later. ADM (A) and RAMP3 (B) gene-expression levels were estimated by quantitative PCR. Shown is the change in expression over time relative to the gene-expression level at 0 h (fold change). Uterine RNA was prepared from three independent experiments and average fold changes and errors are indicated. The *x* axes indicate the time after estrogen administration while the *y* axes indicate fold changes in gene expression. (C) Of the different RAMP genes, only RAMP3 was strongly activated by estradiol. Total uterine RNA was isolated 6 h after injecting 5 μg/kg b.w. estradiol and the change in RAMP gene expression relative to expression in the sesame-oil-injected control uteri was estimated by quantitative PCR. Uterine RNA was prepared from three independent experiments and the average fold changes and errors are indicated. (D) Gene expression of RAMP3 relative to CRLR expression mRNA after estradiol administration was determined by quantitative PCR. The ratio of RAMP3 mRNA to CRLR mRNA was calculated on the basis of the copy numbers of RAMP3 and CRLR mRNA. Approximately equal amounts of mRNA were detected 2 h after estrogen administration. The *x* axis indicates time after estrogen administration while the *y* axis indicates the ratio of RAMP3 mRNA to CRLR mRNA. E2, 17β-estradiol.

essential, the rapid increase of RAMP3 mRNA may affect the properties of the ADM receptor and it appears that the net effect of estrogen exposure is to convert existing CRLR protein into an ADM receptor.

The ER binds to the promoter region of the ADM and RAMP3 genes

Since ADM expression is induced early after estrogen administration, we examined whether the ADM gene

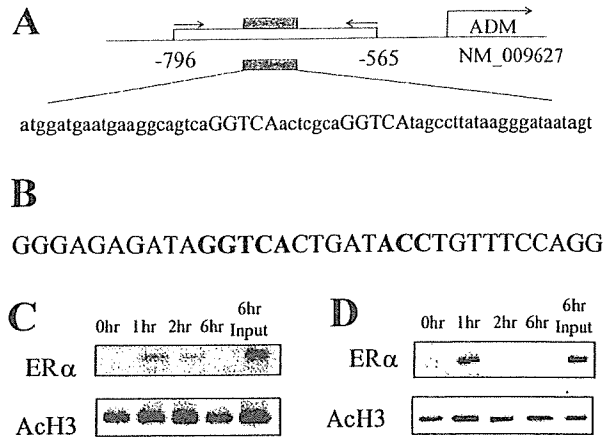

Figure 3 Chromatin immunoprecipitation of the ADM promoter region. (A) Schematic diagram of the ADM promoter region. The ADM gene is indicated on the right. The putative ERE is indicated by a black box. The nucleotide sequence containing the two direct repeats is indicated (the repeats are indicated by capital letters). (B) The open box indicates the region amplified by PCR. The nucleotide sequence of RAMP3 intron containing the putative ERE is indicated (the motif is highlighted in bold). (C) The ADM promoter region was precipitated by anti-ERα antibody. Chromatin immunoprecipitation was performed on mouse uteri obtained 0, 1, 2 and 6 h after treatment with 5 μg/kg b.w. estradiol. At 1 and 2 h after estrogen administration, the ADM promoter region was precipitated by anti-ERα (upper panel). When chromatin immunoprecipitation using anti-acetylated histone H3 (AcH3) antibody was performed, the ADM promoter region was precipitated both before and after estrogen administration (lower panel). Without specific antibodies, no DNA was amplified (data not shown). (D) The RAMP3 intron was precipitated by anti-ERα antibody. Chromatin immunoprecipitation was performed on mouse uteri obtained 0, 1, 2 and 6 h after treatment with 5 μg/kg b.w. estradiol. At 1 h after estrogen administration, the RAMP3 intron was precipitated by anti-ERα (upper panel). When chromatin immunoprecipitation using anti-acetylated histone H3 antibody was performed, the same region was precipitated both before and after estrogen administration (lower panel). Without specific antibodies, no DNA was amplified (data not shown).

can be directly activated by the ER. Analysis of the sequence upstream of the ADM gene revealed direct repeats of the canonical ERE motif between positions −744 and −748 and between positions −756 and −760 from the 5′ end of the ADM gene (NM_009627; Fig. 3A). [The actual distance from the transcriptional start site may differ since the transcriptional start site database (http://dbtss/hgc/jp/) showed that the ADM gene has multiple transcriptional start sites located between positions −9 and −14.] To examine whether the ER can bind to the element *in vivo*, we performed chromatin-immunoprecipitation assays with uterine DNA obtained 0, 1, 2 and 6 h after treatment with 5 μg/kg b.w. estradiol. A DNA fragment containing the direct repeats was precipitated by the anti-ERα antibody only after estrogen administration (Fig. 3C), suggesting that the ER binds to the promoter region of the ADM gene and activates its transcription. Unlike the ligand-dependent binding of the ER to the ADM promoter, anti-acetylated histone H3 antibody precipitated the direct repeat-bearing DNA fragment even before estrogen was added (0 h). Thus the chromatin structure of the ADM gene is open before the addition of ligand and is accessible to the ER when the ligand is administered. Similarly RMAP3 expression was also activated by estrogen. Although ERE could not be found near the transcription start site, a putative ERE was found in the first intron of RAMP3 gene (13 620 bp from the 5′ end of the first intron; Fig. 3B). Chromatin immunoprecipitation confirmed that the DNA fragment containing the putative ERE could be precipitated by anti-ERα only when estrogen was administered (Fig. 3D). Similar to ADM gene, anti-acetylated histone H3 antibody could precipitate the DNA fragment of RAMP3 even before estrogen administration.

The ADM gene is expressed in uterine stroma cells

To examine the location of ADM gene expression in the uterus, we used *in situ* hybridization to detect ADM mRNA in uteri obtained 6 h after stimulation with 5 μg/kg b.w. estrogen. ADM mRNA was mainly detected in the endometrial stroma (Fig. 4). In contrast to the weak signal in unstimulated ovariectomized uteri, the ADM gene was strongly expressed in the stroma of the estrogen-stimulated uteri. Thus estrogen induces ADM gene expression in the stroma only. Interestingly, the ADM gene was not expressed in the myometrium or epithelial cells. A similar expression pattern was observed for RAMP3, namely weak expression in unstimulated uteri and strong expression in the stroma of estrogen-stimulated uteri. The distribution of RAMP3 mRNA in the stroma was similar to that of CRLR mRNA (data not shown). In contrast, RAMP2 expression did not change after estrogen administration, although its expression was limited to the stroma. Thus the temporal (Fig. 2) and spatial (Fig. 4) expression of the ADM and RAMP3 genes correlated closely.

ADM gene is not activated in ERα-null mice

To examine whether ADM gene activation is dependent on ER, we examined the gene-expression profile of ERα and -β-null mice using DNA microarray. As shown in Fig. 5, ADM was activated in wild-type and ERβ-null mice but not ERα-null mice. This result confirmed that ERα is responsible for the gene activation of ADM. Similarly, RAMP3 was also activated in wild-type and ERβ-null mice but not ERα-null mice. These results suggest that both ADM and RAMP3 genes are regulated by ERα but not ERβ.

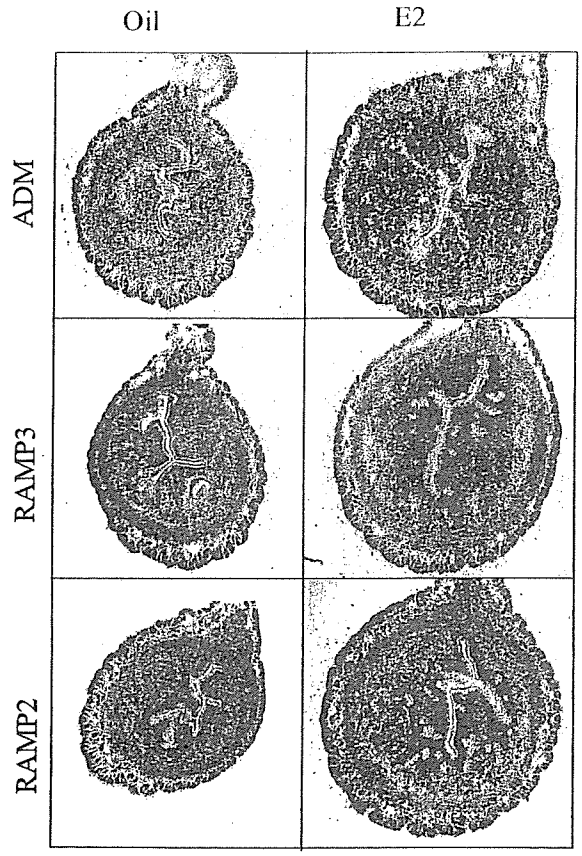

Figure 4 Distribution of ADM, RAMP3 and CRLR mRNAs in estrogen-treated uteri. Ovariectomized mice were given 5 μg/kg b.w. estradiol and their uteri were isolated 6 h later. ADM, RAMP3 and CRLR gene expression was examined by *in situ* hybridization. As a control, mice were injected with sesame oil. The dark-field images were converted to red and merged with bright-field images. E2, 17β-estradiol.

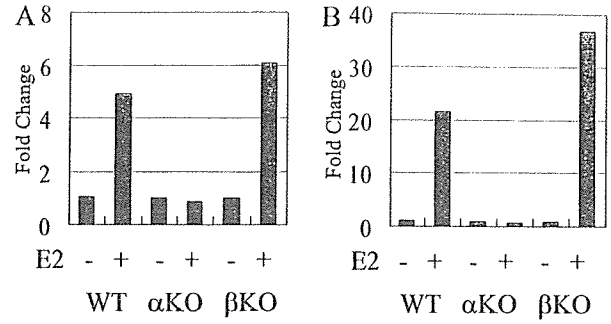

Figure 5 ADM and RAMP3 genes are not activated in ERα-null mice. Female C57BL/6J mice and ERα- and -β-null mice were ovariectomized at 8 weeks of age and injected with either 17β-estradiol (E2, +; 50 μg/kg body weight) or vehicle (−) at 10 weeks of age. The whole uteri were collected 6 h later, and the gene-expression profile was analyzed by DNA microarray. Calculated fold changes are indicated. (A) ADM, (B) RAMP3. WT, wild type; αKO, ERα-knockout; βKO, ERβ-knockout.

Discussion

Since the development of DNA microarray technology, many genes have been listed as being responsive to specific stimuli. However, despite knowing which genes may be activated or repressed by a particular stimulus, the biological significance of this information is still largely lacking. Moreover, many genes identified by DNA microarray analysis have not been validated to respond to the stimulus in question by other methods. In this study, we focused on ADM and RAMP3 genes that were shown by DNA microarray analysis to be activated by estrogen and showed that the ADM and RAMP3 genes are directly regulated by the ER.

ADM was originally identified as a potent vasorelaxant peptide that is produced by pheochromocytoma cells (Kitamura *et al.* 1993*b*). The human form consists of 52 amino acid residues and the mouse and rat forms consist of 50 amino acid residues (Sakata *et al.* 1993). Subsequent studies revealed that ADM is expressed not only in vascular endothelial cells but also in the adrenal gland (Kitamura *et al.* 1993*a*), kidney (Kitamura *et al.* 1993*a*), heart (Perret *et al.* 1993), bone (Cornish *et al.* 1997) and other tissues. Thus ADM is active in the cardiovascular system (Ishiyama *et al.* 1993), the endocrine system (Yamaguchi *et al.* 1995) and the central nervous system (Wang *et al.* 1995). It is also known to be expressed in the reproductive system as its expression in the uterus has been observed; moreover, uterine ADM expression increases during pregnancy (Di Iorio *et al.* 1999, Michishita *et al.* 1999, Thota *et al.* 2003, Upton *et al.* 1997). Furthermore, studies of the relationship between estrogen and ADM expression in the uterus have revealed a positive correlation between estrogen levels and ADM gene expression (Cameron *et al.* 2002, Ikeda *et al.* 2004, Jerat & Kaufman 1998). Thus ADM appears to participate in phyiological processes affecting the uterus.

Many studies on ADM are mainly concerned with its long-lasting activity in physiological processes such as hypotension (Kitamura *et al.* 1995), bone development (Cornish *et al.* 2003) and gestation (Thota *et al.* 2003, Upton *et al.* 1997). With regard to the reproductive system, ADM has been reported to generally act as a relaxant or vasodilator (Makino *et al.* 1999, Yanagita *et al.* 2000). However, the data presented in this paper show that locally expressed ADM can also function in the uterus in a more transient manner, since estrogen-induced ADM gene expression in the uterus only occurs in the stroma over a short period. Interestingly, although ADM, RAMP3 and CRLR are only expressed in the stromal cells, this kind of local effect of ADM has been observed in many tissues (Kato *et al.* 1997, Martinez *et al.* 1997, Nishimura *et al.* 1997, Seguchi *et al.* 1995, Takahashi *et al.* 1997), including the

uterus (Nikitenko *et al.* 2000). Further analysis of ADM signaling is needed to clarify the effects of estrogen on uterine growth.

In the present study, we also demonstrated by chromatin-immunoprecipitation assays that the ADM promoter is recognized by the ER in a ligand (estrogen)-dependent manner. Although we used whole uteri for this analysis, these results appear to reflect the effects of ADM in the stroma, which is abundant in the uterus. Our data show that rapid binding of the ER directly to the ADM promoter may be important for ADM gene activation. A putative ERE was also detected in the RAMP3 gene, though it was found in the first intron of the gene. Chromatin immunoprecipitation confirmed that this region could be recognized by ERα in a ligand-dependent manner.

In summary we have shown that ADM and RAMP3 are genes that are directly activated by the ER, and that ADM and RAMP3 are directly recognized by the ERα. Similarities between the gene-activation patterns of ADM and RAMP3 suggest that a combination of RAMP3 and CRLR functions as an ADM receptor after estrogen administration. Although functional analysis of these genes is essential, the early activation of ADM after estrogen administration suggests that ADM plays important roles in the reproductive system in different ways: first, in processes such as pregnancy, where its involvement and expression are prolonged (Di Iorio *et al.* 1999, Michishita *et al.* 1999, Thota *et al.* 2003, Upton *et al.* 1997), and second, in processes such as estrogen-induced uterine growth, where its involvement and expression are transient.

Acknowledgements

This work was supported in part by a Core Research for Evolutional Science research grant from the Japan Science and Technology Cooperation, a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, a Health Sciences Research Grant from the Ministry of Health, Labour and Welfare of Japan, a research grant from the Ministry of Environment, Japan, and a research grant from New Energy and Industrial Technology Development Organization (NEDO). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 26 July 2005
Accepted 17 October 2005

Journal of Pathology
J Pathol 2006; **209**: 522–531
Published online 25 April 2006 in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/path.1993

Original Paper

PTOV1: a novel testosterone-induced atherogenic gene in human aorta

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Received: 25 October 2005
Revised: 8 February 2006
Accepted: 4 March 2006

Abstract

There are gender differences in the development of atherosclerosis, possibly owing to differences in sex steroid hormone action and/or metabolism. One of the atherogenic effects of testosterone is thought to be androgen receptor (AR)-mediated vascular smooth muscle cell (VSMC) proliferation. However, the detailed mechanism of this effect, particularly the identity of the genes associated with VSMC proliferation, remains largely unknown. Therefore, we first employed microarray analysis and, subsequently, quantitative RT-PCR to analyse RNA expression in AR-positive human VSMCs treated with testosterone in order to detect testosterone-induced genes associated with cell proliferation. We further examined whether the genes identified were involved in cell proliferation using small interfering RNA (siRNA) transfection. Expression of the gene products was then evaluated in human aorta with various degrees of atherosclerosis in order to evaluate the clinical relevance of the findings. Both microarray and quantitative RT-PCR analyses demonstrated marked induction of the human prostate overexpressed protein 1 (*PTOV1*) gene by testosterone in the cell lines: this gene was recently identified as a novel androgen-induced gene involved in prostate tumour cell proliferation. Inhibition of *PTOV1* by transfection of its corresponding siRNA suppressed testosterone-induced cell proliferation. In human aorta, PTOV1 immunoreactivity in the nuclei of neointimal VSMCs was abundantly detected in male aorta with mild atherosclerotic changes compared with female aorta or male aorta with severe atherosclerotic changes. These findings indicate that the *PTOV1* gene is androgen-responsive in VSMCs and that it may play an important role in androgen-related atherogenesis in the human aorta, particularly early atherosclerosis in the male aorta, through regulating proliferation of neointimal VSMCs.

Keywords: vascular smooth muscle cells; androgen receptor; testosterone; cell proliferation; PTOV1

Introduction

There is an important, well-documented, gender difference in coronary heart disease risks, with earlier onset of disease and excess mortality in male subjects [1–3]. Athero-protective effects of oestrogens on vascular structure and function have been proposed as one of the most important mechanisms accounting for this gender difference [4]. On the other hand, an association between androgens and atherosclerosis continues to be disputed. Androgens have been considered to reduce the incidence of ischaemic myocardial disease in men, but they have also been reported to exert atherogenic effects on the human cardiovascular system through promoting plaque formation and enhancing monocyte adhesion to endothelial cells [5–8]. It has been demonstrated that testosterone exerts direct atherogenic effects by promoting cell proliferation through an initial interaction with the androgen receptor (AR) in vascular smooth muscle cells (VSMCs) *in vitro* [9]. However, unlike oestrogens, the possible effects of testosterone on atherogenesis and/or anti-atherogenesis have not been extensively studied. It is therefore important to study the detailed mechanisms of these direct effects of testosterone on the human cardiovascular system.

In this study, we first screened for testosterone-induced genes involved in the proliferation of VSMCs using microarray analysis in cell lines derived from AR-positive human VSMCs. We then confirmed the results by employing other *in vitro* studies. As testosterone induced marked overexpression of *PTOV1* in

these assays, we subsequently examined the levels of expression of PTOV1 protein in VSMCs in samples of the human abdominal aorta obtained at autopsy.

Materials and methods

Vascular smooth muscle cells

Two types of human dedifferentiated VSMCs, ie HUVS-112D (derived from human umbilical cord), and T/G HA-VSMC (derived from human aorta) were commercially obtained from American Type Culture Collection (Manassas, VA, USA) [10,11]. We examined whether these cells expressed AR using an RT/real-time PCR with a light Cycler System using DNA binding dye SYBR Green I, and immunoblotting with AR polyclonal antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), as reported previously [10,12].

GeneChip microarray assay

The VSMCs above were cultured until a sub-confluent state was achieved. The medium was then replaced with fetal bovine serum (FBS)-free and phenol red-free medium to arrest cell proliferation. After 24 h, the medium was replaced again with phenol red-free and FBS-free medium in the presence of testosterone (10 nM) or vehicle (0.1% ethanol). After incubation for 2 h, the cells were subsequently subjected to total RNA extraction for microarray analysis. Isolated total RNA was labelled as described in the Affymetrix (Santa Clara, CA, USA) GeneChip Expression Analysis Technical Manual (revision 3), as previously described [10]. The ratios represent the values up- or down-regulated by 10 nM testosterone treatment compared with control. We independently repeated the same experiment twice. Genes for which the average ratios increased more than 1.5-fold in both experiments using 10 nM testosterone treatment were considered up-regulated via AR when compared with control values [13]. When studying the potential functions of these genes, we used the homepage of the HUGO Gene Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature, accessed 28 March 2006) for further examination of whether any had been previously reported to be involved in cell proliferation and to be associated with androgen effects. In this study, among the genes that were found to be significantly induced by testosterone treatment by microarray analysis, we regarded a gene that was up-regulated, and was known to be associated with both cell proliferation and androgenic effects, as a target gene.

Quantitative real-time PCR

After achieving sub-confluence and following growth arrest states of the VSMCs as described above, the medium was replaced again with phenol red-free and FBS-free F12-K medium with testosterone (10 nM), testosterone (10 nM) with flutamide, an AR-blocker (100 nM), or vehicle. After incubation for 2 h, the cells were subsequently subjected to total RNA extraction for RT/real-time PCR analysis, described previously [10]. mRNA levels for the target gene *PTOV1* were determined in each VSMC as a ratio relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and evaluated as a ratio (%) compared with that of each control cDNA. The analyses with real-time PCR were repeated three times. Table 1 summarizes the primers used [14].

siRNA preparation, transfection, and cell count assay

Small interfering RNAs (siRNAs) corresponding to *PTOV1* (Table 2) were synthesized based on results of a previous report, and were transfected into the VSMCs [15]. These VSMCs were seeded in a 24-well plate at an initial concentration of 50 000 cells/well with F-12K medium containing 2% FBS and were cultured until a sub-confluent status was achieved. The medium was then replaced with phenol red-free and FBS-free medium to arrest cell proliferation. After 24 h, transfection experiments of siRNA for endogenous gene targeting (10 nM or 100 nM) were carried out using RNAiFect™ transfection reagent (Qiagen, Valencia, CA, USA). After transfection, the cells were incubated in phenol red-free medium containing 2% dextran-coated, charcoal-stripped FBS with testosterone (10 nM) or vehicle (0.1% ethanol) for 24 h. We measured the number of cells in each sample as described above with Cell Counting Kit-8 system (Wako, Tokyo, Japan) after incubation for 48 h. We also examined the number of cells treated with

Table 1. Primer sequences used in RT-PCR analysis

cDNA	Sequence	Size (bp)
AR	Forward 5′-CTCACCAAGCTCCTGGACTC-3′ Reverse 5′-CAGGCAGAAGACATCTGAAG-3′	246
GAPDH	Forward 5′-TGAACGGGAAGCTCACTGG-3′ Reverse 5′-TCCACCACCCTGTTGCTGTA-3′	307
PTOV1	Forward 5′-CACCATCCCTCCATGTTGCTG-3′ Reverse 5′-TCTTCATTGGCCTCATCCCC-3′	250

Table 2. Sequences used in siRNA transfection analysis

cDNA	Sequence
PTOV1	Sense r(CAACAAGUUUCUGGCAUGG)dTdT Antisense r(CCAUGCCAGAAACUUGUUG)dTdT
Negative control	Sense r(UUCUCCGAACGUGUCACGU)dTdT Antisense r(ACGUGACACGUUCGGAGAA)dTdT

The target gene in this study (*PTOV1*) was determined by microarray analysis. The sequences of *PTOV1* siRNAs are based on a previous report [15].

transfection of negative control siRNA with scrambled sequences (Table 2), and treated with testosterone (10 nM) or vehicle. In order to evaluate transfection efficiency, we examined relative *PTOV1* mRNAs levels in these cells at 24 h after transfection of the specific siRNAs. The mRNA levels in each VSMC were calculated as a ratio relative to *GAPDH*, and were normalized to the ratio after transfection of negative control siRNA (10 nM).

Quantitative RT-PCR analysis of *PTOV1* mRNA expression in human aorta

Samples of human abdominal aorta were collected at autopsy from patients without a history of hormone replacement therapy. Autopsies were performed on 32 subjects (16 male, 16 female; mean 60.7 ± 3.3 years) in Tohoku University Hospital (Sendai, Japan) within 2 h post mortem. The Ethics Committee at Tohoku University School of Medicine approved the research protocol for this study. Aortic specimens were tentatively classified into the following four groups according to the sex of the deceased patient and degree of atherosclerosis, as previously described: group A = male, mild atherosclerosis, corresponding to groups I–III in the American Heart Association (AHA) classification; group B = male, advanced atherosclerosis, corresponding to groups IV–VI in the AHA classification; group C = female, mild atherosclerosis; and group D = female, advanced atherosclerosis) [10,11]. The distribution of the cases among these groups was as follows: A, 8 cases (mean 44.3 ± 10.6 years); B, 8 cases (mean 71.3 ± 3.7 years); C, 8 cases (mean 52.0 ± 3.9 years); and D, 8 cases (mean 75.0 ± 2.1 years), respectively. For RT/real-time PCR analysis, these specimens were treated according to our previous report [10]. The mRNA levels for *PTOV1* and *AR* in each sample are given as a ratio relative to *GAPDH*, and evaluated as a ratio (%) compared with that of each control cDNA.

Immunohistochemical analysis for PTOV1 protein expression in human aorta

Details of immunohistochemical procedures have been previously described [10,11]. We used immunostaining with diaminobenzidine (DAB) for immunohistochemical analysis of PTOV1 protein (using a monoclonal anti-human PTOV1 antibody; Novocastra Laboratories, Newcastle, UK) and AR (using a monoclonal antibody for human AR; Dako Corporation, Carpinteria, CA, USA). We also used double immunostaining with DAB and Vector Blue as colorimetric reagents, with a combination of monoclonal antibodies for α-smooth muscle actin (α-SMA; Dako Corporation), for macrophages (PG-M1, Dako Corporation), and for leukocytes (human leukocyte common antigen antibody (LCA; Dako Corporation) in adjacent tissue sections. After determining the areas for evaluation by simultaneous observation using a multi-headed light microscope, three authors (YN, TS, and HS) independently evaluated immunoreactivity. Scoring of immunoreactivity was performed based on our previous reports with some modifications [10,16]. When PTOV1 protein was immunolocalized to the cytoplasm, the relative immunoreactivity in each specimen was classified into the following three groups: 2 = more than 50% positive cells; 1 = more than 10% and less than 50% positive cells; and 0 = negative or less than 10% positive cells, respectively [16]. When PTOV1 protein immunoreactivity was detected in the nuclei, the relative immunoreactivity in each specimen was evaluated by the percentage of immunoreactivity, ie the labelling index (LI) [10]. When inter-observer differences were >5%, the three aforementioned authors re-evaluated these discrepant immunostained slides simultaneously using a multi-headed light microscope, and the mean value was obtained.

Statistical analysis

Values for all results were given as the mean ± standard error of the mean (SEM). Results of quantitative RT-PCR, cell count assay, and the relative immunoreactivity for protein in the nuclei were analysed using one-way analysis of variance followed by unpaired *t*-test for comparisons between two groups. Results of immunohistochemistry of cytoplasmic protein were analysed using the χ^2-test. Statistical differences between immunoreactivity for PTOV1 protein and AR were evaluated using Spearman's rank correlation. A p value <0.05 was considered significant in this study.

Results

Characterization of two VSMC cell lines

By RT-PCR analysis, both HUVS-112D cells and T/G HA-VSMC cells expressed *AR* mRNA (Figure 1A). In addition, AR protein was demonstrated in both of these cell lines by immunoblotting analysis (110 kD) (Figure 1B). Relative levels of AR mRNA and protein expression in these cells were approximately 5–10% of those in LNCaP cells that were examined in parallel (data not shown).

Gene chip microarray assay

Table 3 summarizes 11 genes that were up-regulated by testosterone treatment in both types of VSMC in duplicated microarray analysis. Among these genes, human prostate tumour overexpressed protein 1, ie *PTOV1* was detected in both of these VSMCs. Recently, *PTOV1* has been reported to be induced by androgen and to be involved in cell cycle regulation [14,15,17]. *AGTR2* was also reported to be associated with androgenic effects, but it is unknown whether *AGTR2* is involved in cell growth [18]. Therefore we focused our subsequent studies on *PTOV1* as

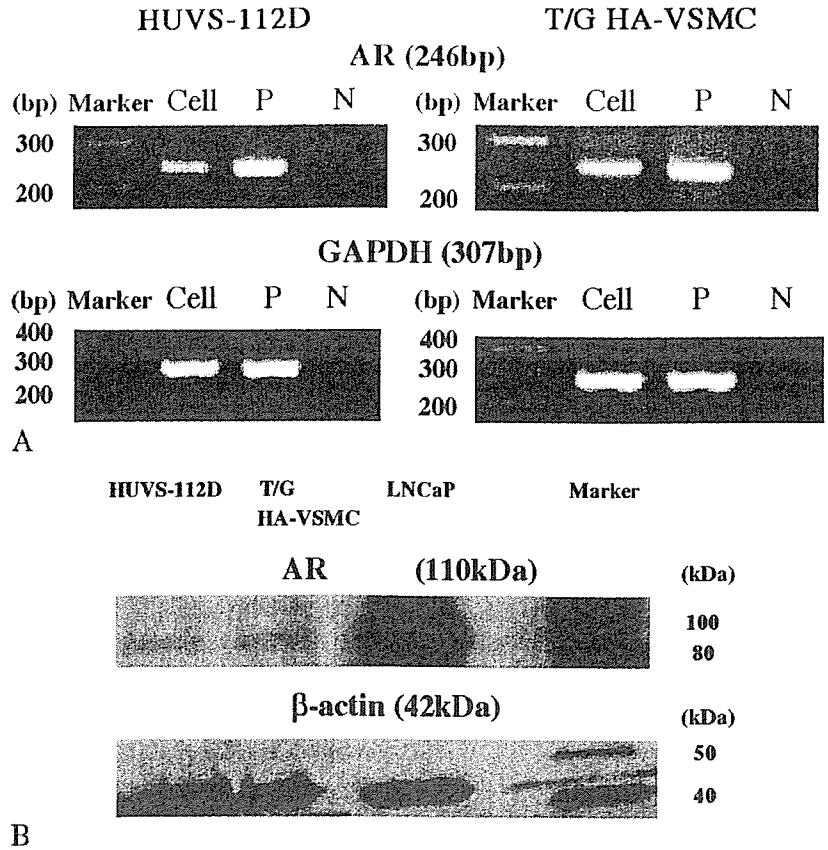

Figure 1. (A) Results of RT/real-time PCR analysis for *AR* and *GAPDH* in two cultured human VSMCs (HUVS-112D, and T/G HA-VSMC), positive controls, and negative controls. Cell = each type of cultured vascular smooth muscle cell; P = positive control (LNCaP prostate cancer cell line); N = negative control (no cDNA), respectively). (B) Immunoblotting analysis of AR and β-actin in HUVS-112D, T/G HA-VSMC, and LNCaP cells. Total protein was extracted, and 60 µg protein from each cell was loaded. Immunoblotting analysis demonstrated both AR (110 kD) and β-actin protein (42 kD) in all cells

Table 3. Ratios of gene expression determined by GeneChip microarray analysis after testosterone treatment of cultured VSMCs for 2 h

Gene symbol	HUVS-112D	T/G HA-VSMC	Function	Association with androgen (reference)
PAK7	3.9	3.6	Neurite development	Unknown
PIK3R4	1.7	3.3	Cell signalling	Unknown
CELSR1	1.9	3.1	Cell adhesion	Unknown
CACNA1G	2.5	2.9	Calcium channel	Unknown
AGTR2	2.2	2.5	Regulator of aldosterone secretion	Koike *et al* [18]
INVS	2.7	3.6	Renal tubular development	Unknown
GPR77	3.5	2.6	Cell signalling	Unknown
CASP10	3.3	2.8	Apoptosis	Unknown
AP4S1	2.1	2.5	Formation of cell structure	Unknown
T1A-2	1.7	2.7	Membrane glycoprotein	Unknown
PTOV1	1.8	2.0	Cell growth/mitogenesis	Benedit *et al* [14]

'Ratios' represent the mean ratios of expression levels of each gene mRNA in duplicate experiments compared with control.

an androgen-responsive gene possibly involved in the proliferation of human VSMCs.

PTOV1 mRNA expression in VSMCs after androgen treatment

Testosterone significantly increased *PTOV1* mRNA levels in AR-positive VSMCs compared with controls in both of these cell lines ($p < 0.05$) (Figure 2). However, testosterone with flutamide, an AR-blocker (100 nM), did not increase its mRNA expression in either of these cells ($p < 0.05$) (Figure 2).

PTOV1 siRNA transfection and cell proliferation assay

Quantitative RT-PCR analysis demonstrated that *PTOV1* mRNA levels were decreased in a dose-dependent manner in the cells transfected with *PTOV1* siRNAs (Figure 3A). After transfection of negative

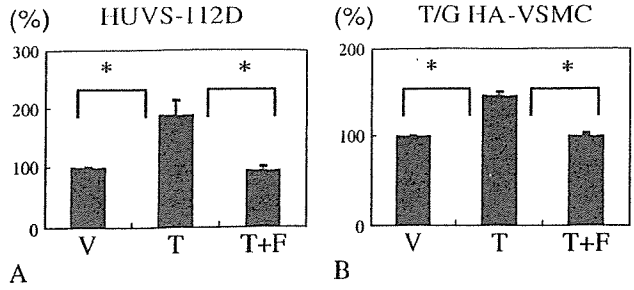

Figure 2. Results of RT/real-time PCR analysis for *PTOV1* in HUVS-112D (A) and T/G HA-VSMC cells (B) among cells treated with vehicle (V) (control), testosterone (T) alone (10 nM), and T (10 nM) with flutamide (F), an AR-blocker (100 nM), respectively after 2 h (*$p < 0.05$)

control siRNA (10 nM), testosterone promoted cell proliferation significantly in both of these cell lines ($p < 0.05$) (Figure 3B). However, testosterone with transfection of *PTOV1* siRNA (10 nM and 100 nM) did not increase cell proliferation in these two cell lines (Figure 3B).

PTOV1 mRNA expression in human aorta

The results of RT/real-time PCR analysis demonstrated the presence of specific single bands for *AR* and *PTOV1* in human aorta (Figure 4A). The relative abundance of *PTOV1* mRNA was significantly greater in male aorta with a mild degree of atherosclerotic changes (group A) than in those of other groups (groups B, C, and D)($p < 0.05$) (Figure 4B). The relative abundance of *AR* mRNA was also significantly greater in male aorta with a mild degree of atherosclerotic change (group A) than in male aorta with a severe

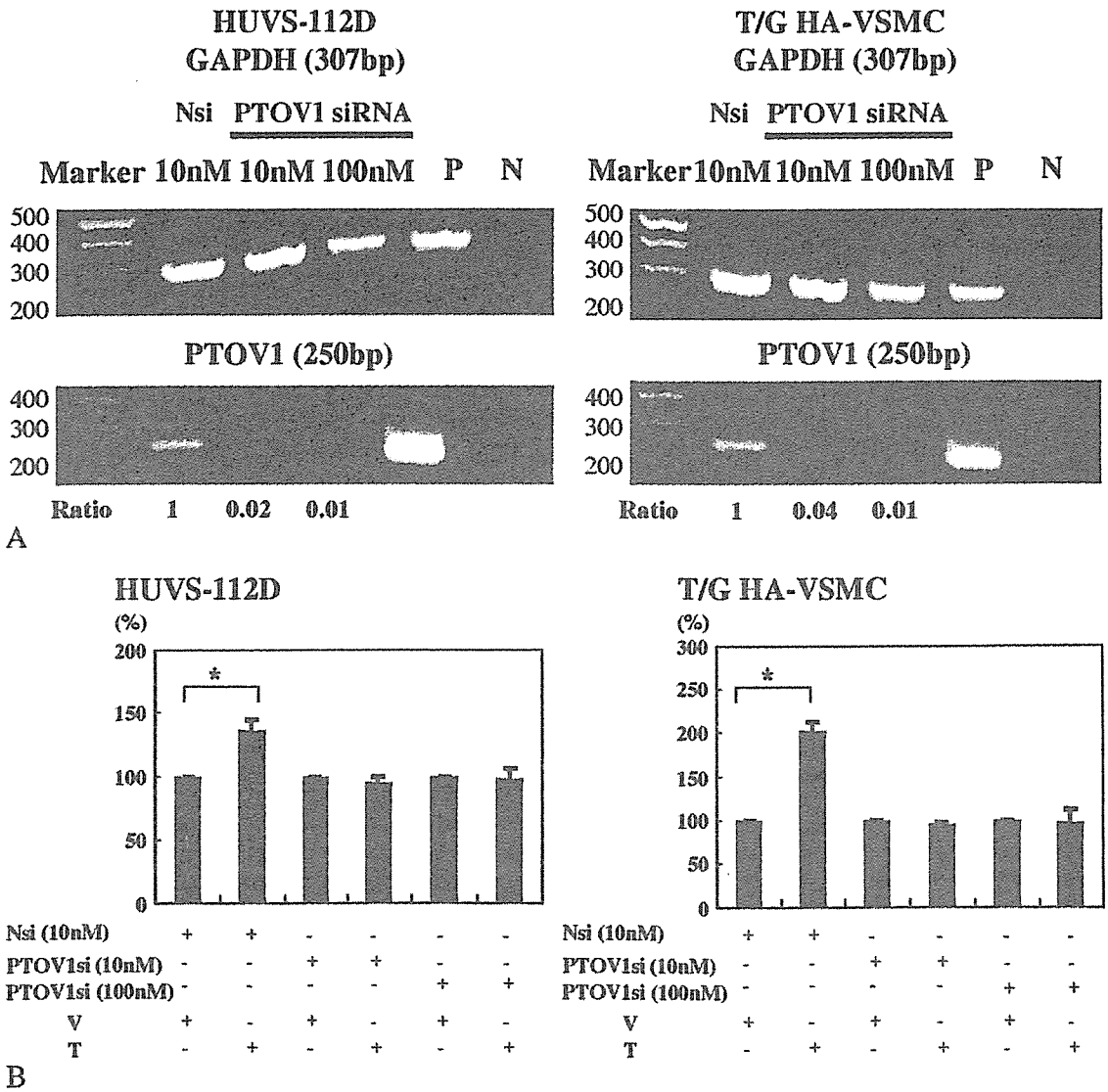

Figure 3. (A) Expression of *PTOV1* and *GAPDH* mRNAs at 24 h after transfection of *PTOV1* siRNA (10 nM or 100 nM) or negative control siRNA (Nsi) (10 nM) in HUVS-112D and T/G HA-VSMC cells detected by real-time PCR, respectively. *GAPDH* mRNA expression was monitored as an internal control. The ratio of *PTOV1*/*GAPDH* was calculated and values were normalized to the ratio obtained from the negative control transfection of Nsi (10 nM). P = positive controls (LNCaP prostate cancer cell lines); N = negative controls (no cDNAs), respectively. (B) The relative levels of cell numbers in HUVS-112D and T/G HA-VSMC cells among cells treated with vehicle (V) (0.1% ethanol) and testosterone (T) alone (10 nM) after transfection of *PTOV1* siRNA (PTOV1si) (10 nM or 100 nM) or negative control siRNA (Nsi) (10 nM) (*$p < 0.05$)

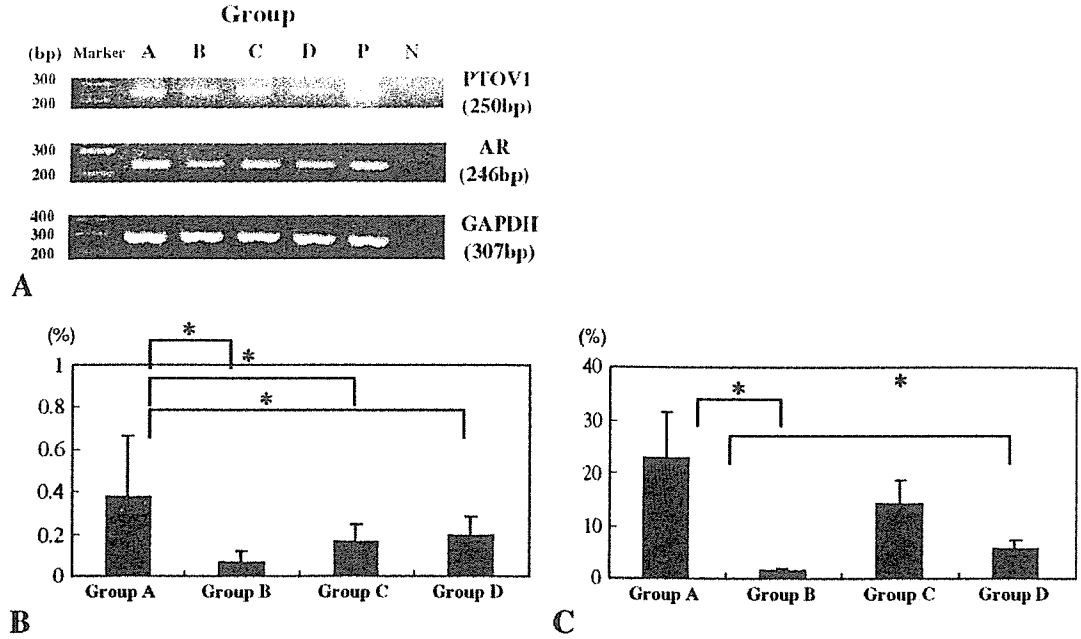

Figure 4. (A) Results of RT/real-time PCR analysis for *PTOV1* in human aortas. Bands for PCR products were detected as specific single bands (246 bp for *AR*, 250 bp for *PTOV1*, and 307bp for *GAPDH*). The amplified products were run on a 2% agarose gel stained with ethidium bromide. Representative photographs for these RT/real-time PCR gene products are illustrated. A = aorta from a 38-year-old man with mild atherosclerotic change; B = aorta from a 72-year-old man with severe atherosclerotic change; C = aorta from a 45-year-old woman with mild atherosclerotic change; D = aorta from a 76-year-old post-menopausal woman with severe atherosclerotic change; P = positive controls; N = negative controls. (B) Results for *PTOV1* mRNA expression levels (*$p < 0.05$). (C) Results for *AR* mRNA expression levels (*$p < 0.05$)

degree of atherosclerosis (group B) and in female aorta with a severe degree of atherosclerosis (group D) ($p < 0.05$) (Figure 4C).

Immunohistochemistry for PTOV1 in human aorta

PTOV1 protein was expressed in both the nucleus and the cytoplasm of VSMCs in each group examined (Figures 5 and 6). AR protein was expressed in the nuclei of VSMCs in each group (Figures 5 and 6). However, none of the LCA- or PG-M1-positive cells demonstrated any PTOV1 immunoreactivity (Figure 5). The relative levels of PTOV1 immunoreactivity in the nuclei of neointimal VSMCs were significantly higher in male aorta with a mild degree of atherosclerotic change (group A) than in those of the other groups examined (groups B, C, and D) (Figure 6A). In addition, AR-positive cells in the neointima were also significantly more abundant in male aorta with a mild degree of atherosclerotic changes (group A) than in those of the other groups (groups B, C, and D) ($p < 0.05$) (Figure 6E). There was also a significant positive correlation between AR and PTOV1 immunoreactivity in the nuclei of VSMCs in the neointima ($p < 0.05$) (data not shown). AR-positive cells in the tunica media were significantly more abundant in male aorta with a mild degree of atherosclerotic change (group A) than in male aorta with a severe degree of atherosclerosis (group B) and in female aorta with a mild degree of atherosclerosis (group C) ($p < 0.05$) (Figure 6F). However, there were no significant differences in PTOV1 immunoreactivity in the cytoplasm of cells in the neointima or in the nucleus and/or cytoplasm of cells in the tunica media among these groups (Figure 6B, C, and D).

Discussion

In our present study, results of both microarray and quantitative RT-PCR analyses all indicated that *PTOV1* is one of the genes induced by testosterone via AR-dependent pathways in cultured human VSMCs. In addition, siRNA analysis demonstrated that *PTOV1* is involved in AR-mediated VSMC proliferation. Results of both quantitative RT-PCR and immunohistochemical studies in human aorta obtained at autopsy further demonstrated that PTOV1, as well as AR, detected in the nuclei of neointimal VSMCs was abundant in relatively young male aorta at an early stage of atherosclerosis.

PTOV1 has been known to be involved in stimulation of cell proliferation [14,15,17]. This gene is a mitogenic factor that shuttles between nucleus and cytoplasm in a cell cycle-dependent manner in prostate carcinoma cells [14,15,17]. In addition, *PTOV1* overexpression induced cell proliferation and facilitated entry of prostate carcinoma cells into the S phase [14,15,17]. Therefore, these findings all indicated that *PTOV1* may play a very important role in the proliferation of VSMCs. However, it is also true that other atherogenic effects on human VSMCs, such as promotion of PDGF-induced VSMC proliferation,

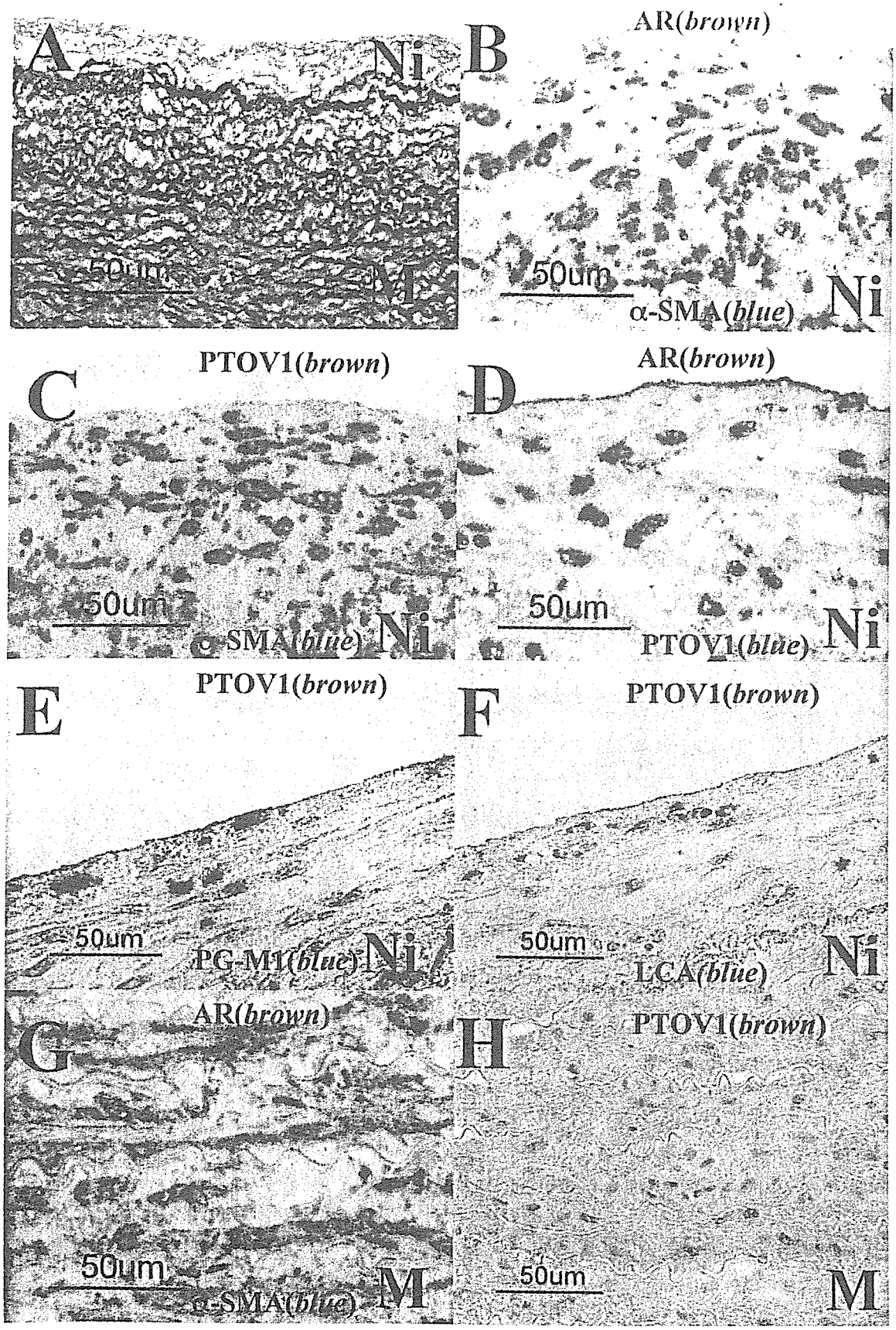

Figure 5. Modified Masson Goldner's stains (A), double-immunohistochemical staining for AR and α-muscle actin (α-SMA) (B), for PTOV1 and α-SMA (C), for AR and PTOV1 (D), for PTOV1 and PG-M1 (E), for PTOV1 and leukocyte common antigen (LCA) (F) in the neointima, double-immunohistochemical staining for AR and α-SMA (G) and immunohistochemical staining for PTOV1 (H) in the media of an abdominal aorta specimen obtained from a 38-year-old man with a mild degree of atherosclerosis (group A). Immunopositive cells appear brown as a result of DAB colorimetric reaction and blue as a result of Vector Blue colorimetric reaction. Double-immunopositive cells are confirmed. Ni = neointima; M = media

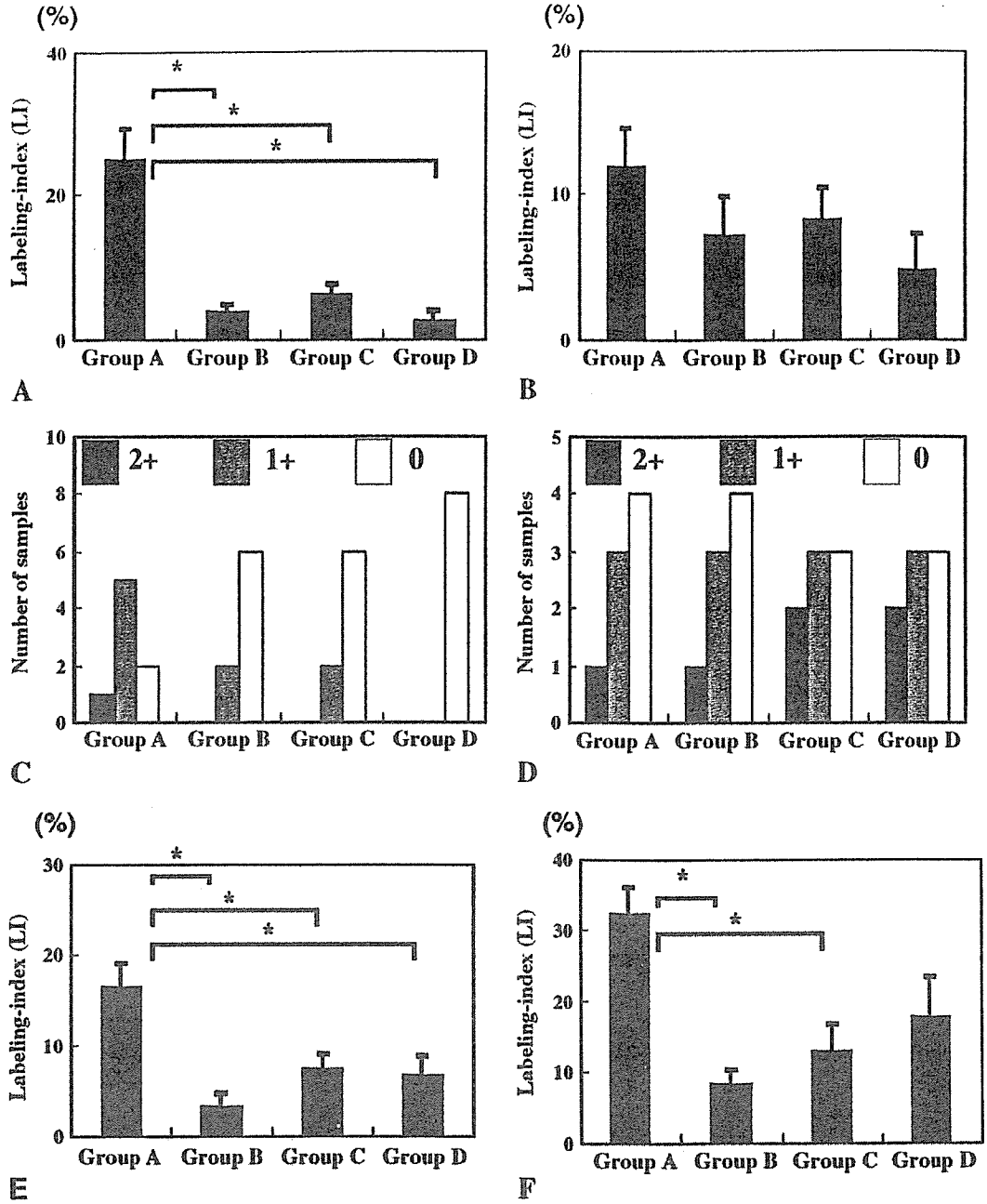

Figure 6. (A, B) The relative immunoreactivity of PTOV1 in the nuclei of VSMCs in the neointima (A) and media (B) was evaluated by the labelling index (LI) in each group (0–100), respectively. Data are the mean ± SEM. $^*p < 0.05$, a significant difference between two groups. (C, D) The relative immunoreactivity of PTOV1 in the cytoplasm of VSMCs in the neointima (C) and media (D) was evaluated by the percentage of positive cells (0, 1+, and 2+) in each group, respectively. (E, F) The relative immunoreactivity of AR present in the nuclei of VSMCs in the neointima (E) and media (F) was evaluated by the LI in each group (0–100), respectively. Data are the mean ± SEM. $^*p < 0.05$, a significant difference between two groups

have been reported as the mechanism for androgen-induced effects [19,20]. Therefore, further investigations are required to clarify how these pathways interact in exerting androgenic effects on VSMC proliferation in the human vascular system. Our present siRNA study demonstrated that *PTOV1* may be involved in testosterone-induced VSMC proliferation. However, further investigation is required to clarify the correlation between PTOV1 expression and testosterone-induced VSMC proliferation by reconstituting *PTOV1* expression after transfection of *PTOV1* siRNA.

Quantitative RT-PCR analysis in our present study also demonstrated that flutamide, an AR-blocker, suppressed androgen-induced *PTOV1* mRNA expression. The chromosomal region where *PTOV1* is located, 19q13.3–13.4, has also been demonstrated to harbour a large number of genes whose expression is modulated by androgens [14]. In addition, the expression of *PTOV1* was reported to be induced by exposure to androgens in LNCaP, an androgen-dependent prostate carcinoma cell line [14,17]. Therefore, these findings all indicate that *PTOV1* should also be considered one of the testosterone-induced genes in AR-positive VSMCs.

We also demonstrated that PTOV1 immunoreactivity in the nuclei of neointimal VSMCs was abundant in relatively young male aorta associated with early stage atherosclerosis. High levels and nuclear localization of PTOV1 have also been associated with cell proliferation in prostate carcinoma cells [14,17]. Neointimal VSMCs are, therefore, considered to play very important roles in the development of atherosclerosis in humans, particularly at an early stage, compared with VSMCs in the tunica media [10,11]. Therefore, higher expression of PTOV1 in these VSMCs is possibly related to the development of atherosclerosis. Levels of PTOV1 and AR were higher in male aorta with mild atherosclerosis than in female aorta with mild atherosclerosis. Men are generally considered to have a higher risk of developing cardiovascular disease than similarly aged women because of prolonged exposure to higher androgen concentrations [19,21]. It has also been shown recently that androgens upregulate atherosclerosis-related genes in macrophages from men, but not from women, which reflects the complexity of gender-related atherogenesis [19,22].

Our present study also demonstrated that the relative abundance of AR and PTOV1 in neointimal VSMCs was significantly higher in younger male aorta with mild atherosclerotic changes than in male aorta with severe atherosclerotic changes. However, these findings appear to contradict the hypothesis that, if PTOV1 is induced by androgens and implicated in androgenic effects on atherosclerosis, its expression should be higher in male aorta with more severe atherosclerosis than in male aorta with mild atherosclerosis owing to a longer exposure to elevated serum testosterone levels. There are two possible reasons for this: firstly, decreased AR and PTOV1 expression in the neointima of male aorta with severe atherosclerosis may be induced by the age-related decrease in serum testosterone levels [23]; and, secondly, when neointimal formation progresses, VSMCs with AR expression become less abundant than those without AR and these cells are therefore not necessarily influenced by androgenic atherogenic effects. Therefore, PTOV1 expression in the neointimal VSMCs in the aortas of men with high serum androgens levels may be associated with the androgen-induced onset of atherosclerosis; this may be important for formation of the neointima in the early stages of atherogenesis in the male aorta. However, recently, low concentrations of testosterone have been associated with an increased risk of cardiovascular disease in men [24]. Androgens are also known to be a coronary vasodilator, and a study of postmenopausal women demonstrated that endogenous androgens correlated inversely with carotid neointimal thickness, which suggests that androgens have potential beneficial effects on the human vascular system [19,25,26]. These different effects of androgens may depend on differences in the androgen-responsive genes induced, but further investigations are required to clarify possible direct androgenic effects on the human cardiovascular system.

In summary, *PTOV1* is considered to be one of the testosterone-induced genes involved in AR-mediated stimulation of VSMC proliferation in the aortic neointima and may play important roles in androgen-related atherogenesis in the male human aorta.

Acknowledgements

This work is in part supported by Health and Labour Sciences Research Grants for Risk Analysis Research on Food and Pharmaceuticals (H13-Seikatsu-013) from the Ministry of Health, Labour and Welfare of Japan. In addition, we thank Miss Naomi Kanai for technical assistance.

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