Progesterone Receptor Subtypes in Vascular Smooth Muscle Cells of Human Aorta

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Abstract. Progesterone is involved in various functions of the cardiovascular system, including those of vascular smooth muscle cells (VSMCs) via progesterone receptor (PR). Progesterone has also been postulated to be involved in inhibition of VSMC proliferation via PR. However, the details of PR expression have remained largely unknown in human cardiovascular VSMCs. Therefore, we first examined the relative levels of PR isoform (PR-A and PR-B) expression in VSMCs, using both immunohistochemistry and quantitative RT-PCR analysis. PR-B was equally expressed between male and female aorta, but PR-A was more abundant in female than in male aorta. This finding demonstrated that the status of PR subtype expression was associated with the difference of genders.

Key words: Vascular smooth muscle cells, Human aorta, Progesterone receptor, Immunohistochemistry, RT/real-time PCR

THE significance of hormone replacement therapy (HRT) with estrogens in combination with progesterone still remains controversial [1–5]. One of the reasons for this controversy involves the possible roles which progesterone plays in the human cardiovascular system (CVS), especially with relation to its potential to regulate numerous factors which influence atherosclerotic processes; affect circulating levels of various lipids [6], cause expression of coagulation and thrombolytic proteins expression [7–9]; and inhibit vasorelaxation [10, 11]. However, it is also conjectured that one important anti-atherogenic action of progesterone is its inhibition of cell proliferation of vascular smooth muscle cells (VSMCs) [12–14]. Therefore, it becomes important to examine this possible mechanism of anti-atherogenic effect of progesterone on VSMCs in human CVS.

The effects of progesterone on VSMCs are considered to take place through its interaction with progesterone receptor (PR) present in these cells. Two isoforms of PR have been identified, PR-A and PR-B, which are 94 kDa and 114 kDa in size, respectively (Fig. 1) [15, 16]. The PR-B isoform is a full-length receptor, whereas the PR-A isoform lacks 164 amino acids in the N-terminus of the PR-B isoform. Both PR-A and PR-B are derived from transcripts initiated from two distinct promoters within a single-copy of the PR gene [16]. However, the relative abundance of PR isoforms, PR-A and PR-B, has not been examined in VSMCs of human aorta. Therefore, in this study, we examined the relative abundance of PR subtypes in VSMCs of human aorta using immunohistochemistry and RT-PCR analysis in order to evaluate the possible biological significance of PR subtypes abundance in human aorta and its diseases.

Materials and Methods

Specimens

Human abdominal aorta were collected at the time of autopsy performed in Tohoku University Hospital,
Sendai, Japan, within three hours postmortem from 50 subjects (20 male, 10 pre-menopausal female, 20 post-menopausal female, mean 53.7 ± 3.4 years old). The research protocol for this study was approved by the Ethics Committee of Tohoku University School of Medicine. The classification between pre-menopausal and post-menopausal status was due to the difference of serum progesterone concentration. For RT/real-time polymerase chain reaction (PCR) studies, we could examine only whole aorta due to the difficulty incurred in trying to separate the neointima from the media by macroscopic examination. The distribution of the cases among these groups examined was summarized as follows: total 39 subjects (15 male, 9 pre-menopausal female, 15 post-menopausal female, mean 56.1 ± 3.9 years old). Fresh tissues were not available for examination in all the cases. These specimens were collected from the areas adjacent to the specimens obtained for histopathological studies. In addition, the degrees of atherosclerosis of samples were evaluated according to a previous report [17]. The adventitia and fat tissues surrounding the aorta were immediately and carefully removed using clean surgical scissors and forceps at the time of autopsy. Following this procedure, these specimens were immediately frozen in liquid nitrogen and stored at −80°C until use.

**Real-time Polymerase Chain Reaction (PCR)**

Total RNA was extracted by homogenizing frozen tissue samples in 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA) followed by phenol-chloroform phase extraction and isopropanol precipitation. All RNA samples were quantified by spectrophotometry and stored at −80°C until processed for reverse transcription (RT). The SUPERSCRIPT™ Preamplification system RT kit (Gibco-BRL, Grand Island, NY) was employed in the synthesis and amplification of complementary DNA (cDNA). cDNA was synthesized from total RNA (2 μg) using 25 ng/μL Oligo (dT)12–18 Primer (Life Technologies, Inc., Gaithersburg, MD) on a PTC-200 Peltier Thermal Cycler DNA Engine (MJ Research, Inc., Watertown, MA). To test for the presence of genomic DNA contamination, we performed the RT step in the absence of SUPERSCRIPT™ II RNase H Reverse Transcriptase (Gibco-BRL) followed by PCR. RT-PCR products lacking reverse transcriptase in the initial RT step were run on an ethidium-bromide stained 2% agarose gel. No band was detected in these samples (data not shown). The resulting cDNA was used as a template for real-time PCR. Real-time PCR was carried out with Light Cycler System (Roche Diagnostics GmbH, Mannheim, Germany) using DNA binding dye SYBR Green I (Roche Diagnostics GmbH) and Tag DNA polymerase (Life Technologies, Inc.). Each primer is shown Table 1. The fluorescence
The intensity of the double-strand specific SYBR Green I, which reflects the amount of formed specific PCR products, was read by LightCycler. Following PCR, these products were resolved on a 2% agarose ethidium bromide gel. Images were captured with Polaroid film under UV transillumination. In initial experiments, PCR products were purified and subjected to direct sequencing (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 310 Genetic Analyzer, Perkin-Elmer Corp., PE Applied Biosystems, Foster City, CA) to verify amplification of the correct sequences. As a positive control, placenta or adrenal gland was used. Negative control experiments lacked cDNA substrate to check for the presence of exogenous contaminant DNA. No amplified products were observed under these conditions. The mRNA levels of both PR-AB and PR-B in each case are summarized as a ratio of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and evaluated as a ratio (%) compared with that of each standard cDNA. The PCR standards were made for each PR and GAPDH consisted of known numbers of molecules of PCR product purified using a gel extraction kit (Qiagen, Chatsworth, CA) and quantified spectrophotometrically.

### Primary antibodies

The primary antibodies used in the present study were hPRa7 for PR-A and hPRa2 for PR-B (Neomarkers, CA). hPRa7 and hPRa2 were both monoclonal antibodies raised in mouse against PR isoforms obtained from human endometrial carcinoma (EnCa 101). These antibodies were utilized in a previous study [18]. The hPRa7 antibody employed in this study recognized both PR-A and PR-B in immunoblot analysis [19]. However, Mote et al. reported that hPRa7 did not recognize PRB in their immunohistochemistry study of fixed tissues even after antigen retrieval, as evidenced by the absence of immunostaining for this antibody in the PR-B-expressing MDA-MB-231/PR-B cell line [20]. This may be due to the inaccessibility of the epitope on PR-B recognized by hPRa7 in 10% formalin-fixed and paraffin-embedded tissue specimens [20], which, in turn, may be due to an alteration in the conformation of the molecule such that the hPRa7 epitope is located in a position that reduces its accessibility during immunohistochemistry. The hPR-7 antibody was used at a dilution of 1:100 and the hPR-2 antibody at 1:200.

### Immunohistochemistry

Immunohistochemistry was performed using the streptavidin-biotin amplification method employing a Histofine Kit (Nichirei, Tokyo, Japan), which has been previously described in detail [18]. We also utilized double immunostaining with DAB and Vector-blue for these proteins and α-smooth muscle actin (α-SMA), respectively, to further characterize PR positive cells in the human aorta in immunopositive cases. In addition, we also examined whether these proteins were expressed in endothelial cells and macrophages in adjacent tissue sections. Hematoxylin-eosin and modified Masson-Goldner method were used to identify the border between media and neointima.

### Scoring of immunoreactivity

We performed H-score, which evaluates the degree of immuno intensity, as well as the number of immunoreactive PR-A and PR-B cells in VSMCs present in the aortic neointima and media. The H-score was generated by adding together 3 × % strongly stained nuclei, 2 × % moderately stained nuclei, and 1 × % weakly stained nuclei, thereby producing a possible range of 0 to 300 [21]. After determining the areas of evaluation by simultaneous observation using a multi-headed light

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**Table 1. Oligonucleotide primer sequences used for RT-PCR analysis**

<table>
<thead>
<tr>
<th>mRNA</th>
<th>primer</th>
<th>sequence</th>
<th>GeneBank/EMBL Accession number</th>
<th>Nucleotide number</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR-AB</td>
<td>Sense</td>
<td>TGGAAAGAAATGACTGCATCG</td>
<td>NM_000926</td>
<td>3266–3461 (Exon 3–4)</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>TACCTGGTTCCTTTCAATTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR-B</td>
<td>Sense</td>
<td>ACACCTTGCGCTGAAGTTTCG</td>
<td>NM_000926</td>
<td>1582–1778 (Exon 1–1)</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CTGCCCTTTCTGAGGGGACT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense</td>
<td>TGACGGGAAGCTACCTGG</td>
<td>M33197</td>
<td>731–1038 (Exon 11–12)</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>TCCACCACTCTGTCATGA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
microscopy, three blind authors (Y.N., T.S., and H.S.) independently evaluated 100 VSMCs. When inter-observer differences were less than 5%, the mean value was determined as H-score. When inter-observer differences were more than 5%, the three above authors using the multi-headed light microscopy reevaluated immunostained slides simultaneously, and the mean value was obtained.

**Statistical analysis**

Values for the results were presented as mean ± standard error of means (SEM). For comparisons between two groups, we used one-way analysis of variance. In order to examine the correlation of the two factors, Spearman’s correlation coefficient was used. *P*-value less than 0.05 were considered significant in this study.

**Results**

**RT-PCR analysis for PR and PR-B mRNA expression**

Relative abundance of PR mRNA determined by real-time PCR analysis was higher in pre-menopausal and post-menopausal female aorta (1.1 ± 0.3%, 2.2 ± 0.8%, respectively) than in male aorta (0.3 ± 0.1%) (*P*<0.05) (Fig. 2). In addition, relative mRNA abundance of PR-B tended to be higher in pre-menopausal and post-menopausal female aorta (0.34 ± 0.17%, 0.22 ± 0.09%, respectively) than in male aorta (0.15 ± 0.11%), but the differences did not reach statistical significance (Fig. 2). Relative mRNA abundance for these PR subtypes in both male and female aorta determined by real-time PCR analysis was not correlated with the degree of atherosclerosis or age in these specimens (data not shown).

**Immunohistochemistry**

Both PR-A and PR-B immunoreactivity were detected in the nuclei of VSMCs in all the specimens exam-
ined. In addition, a low level of expression of both PR subtypes was detected sporadically in endothelial cells (data not shown). Fig. 3 shows some representative illustrations of an abdominal aorta specimen obtained from a pre-menopausal woman (45 year-old) (A, B), those of a post-menopausal woman (76 year-old) (C, D), and those of a man (38 year-old) (E, F). Double immunohistochemical staining for PR subtypes and

![Fig. 3](image)

**Fig. 3.** Double immunohistochemical staining for α-SMA and PR-A (A) (C) (E), and for α-SMA and PR-B in media (B) (D) (F) of an abdominal aorta specimen obtained from autopsy.

(A, B) an abdominal aorta specimen obtained from a 45 year-old pre-menopausal woman.
(C, D) an abdominal aorta specimen obtained from a 76 year-old post-menopausal woman.
(E, F) an abdominal aorta specimen obtained from a 38 year-old man.

Cells positive for PRs appear brown as a result of DAB colorimetric reaction. Cells positive for α-SMA appear blue as a result of Vector-blue colorimetric reaction. Double immunopositive cells are confirmed, respectively. Original magnification, ×400 for double immunohistochemical staining.
alpha-SMA demonstrated the presence of PRs in VSMC media positive for alpha-SMA. Immunoreactive PR-A protein was much more markedly expressed in VSMCs in female than in male, but immunoreactive PR-B protein was not different between female and male. There were significant differences among the expression levels of PR-A (P<0.05) (Table 2). The number of PR-A positive VSMCs was higher in pre-menopausal and/or post-menopausal female aorta (49 ± 6 H-score, 61 ± 8 H-score, respectively) than in male aortas (36 ± 6 H-score) (Table 2). However, no significant positive correlation was detected among the expression levels of PR-B. In addition, immunoreactivity of both PR-A and PR-B was not significantly correlated with the degrees of atherosclerotic changes (Table 2).

### Discussion

In the present study, both subtypes of PRs were detected in VSMCs of the human aorta. In addition, the relative abundance of PR-A appeared to be more prevalent in the female aorta than in the male aorta.

Analysis of PR subtypes can provide new insights into the possible biological roles of progesterone. Both isoforms have been reported to function as ligand-activated transcription factors, but they are not always equal in their functional properties and progesterone actions [22]. PR-B is, in general, transcriptionally more active than PR-A, but when compared to PR-A, PR-B activity is also cell-specific [23, 24]. In addition, the PR-A isoform has been demonstrated to repress the transcriptional activities of other steroid hormone receptors [25]. Therefore, the relative levels of PR-A and PR-B within target cells are considered to contribute to the nature and magnitude of functional responses to progesterone in these cells. Previous studies on PR expression demonstrated that the relative abundance or expression of PR-A and PR-B in uterine malignant endometrial carcinoma was associated with the biological behavior and/or malignant grades of the tumor to some extent [26, 27]. In contrast, in non-neoplastic tissue, the association between the relative abundance or expression of both PRs and its biological significance has yet to be examined in detail. In our present study, the relative level of PR-A expression was more abundant in VSMCs of the female aorta than in those of the male aorta. But the relative level of PR-B expression was not different between male and female aortas. PR-A has been previously demonstrated to repress the transcriptional activities of other steroid hormone receptors, including estrogen receptor and PR-B [28, 29]. In pre-menopausal female subjects, the relatively high concentrations of progesterone are supplied from the ovary. On the other hand, in this study, relative amounts of PR-A in the post-menopausal aorta were also higher than that in male aorta. This finding suggests that in female aorta the relative abundance of PR-A was not necessarily correlated with circulating progesterone concentration, and that in post-menopausal female aorta progesterone derived from adrenal glands exerts its effects despite its lower circulating concentration. On the other hand, two types of variant PR transcripts specific to human VSMCs have been detected, which were present in pre-menopausal women but absent in post-menopausal women [30]. The complex tissue-specific effects of progesterones may be altered by the status of the heterogeneous PRs expression, including the wild PR-A, PR-B, and the variant PRs. Therefore, further investigation is required to clarify how these subtypes of PRs may modulate biological effects of progesterone in VSMCs.
PROGESTERONE RECEPTOR IN HUMAN AORTA

References


