Intrinsic ANG II type 2 receptor gene polymorphism 1675 G/A modulates receptor protein expression but not mRNA splicing

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The association of an intron 1 AT2 polymorphism, designated as 1675 G/A or -1332G/A based on its location relative to transcription or translation start, with myocardial structure and cardiac disease has been investigated in several studies (3, 15, 22, 24, 37). The A allele was associated with higher left ventricular mass in young male Bavarian untreated hypertensive men and with a greater cardiovascular risk in 2,579 healthy UK men (22). In contrast, the G allele was found to be associated with higher left ventricle (LV) mass in 125 male UK patients with systemic hypertension (3). In the European Project on Genes in Hypertension, different associations of the AT2 polymorphism with LV mass were found depending on sodium excretion in males but not in females (24). In the

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Glasgow Heart Scan Old study the A allele was associated with left ventricular hypertrophy in males and recurrent coronary ischemia and myocardial infarction in elderly females (15). Until now, it could only be speculated which of the AT2 alleles may be associated with a higher AT2 expression, and the molecular mechanisms underlying these putative differences in receptor expression have been obscure.

Because it was suggested that the intronic AT2 1675 G/A polymorphism (PM) might affect pre-mRNA splicing (30), the aim of the present work was to verify this hypothesis or to find alternative explanations for the observed phenotypes. For this purpose, we used myocardial samples from explanted human hearts and transgenic cell cultures models.

**MATERIALS AND METHODS**

**Analysis of the Effect of the AT2 1675 Genotype on the Myocardial AT2 Splice Pattern**

In a previous study (44), we used RT-PCR to analyze the myocardial AT2 splice pattern of 14 explanted failing hearts from patients who had undergone orthotopic heart transplantation at the German Heart Institute (see Table 1 for patient data). Twelve of these patients were now genotyped using single stranded conformation polymorphism (SSCP) PCR and nucleotide sequencing of the PCR products as described previously (9, 37). The Declaration of Helsinki was observed throughout the study.

**Analysis of the Effect of the AT2 PM in Transfected Human and Rodent Cells**

**Cell culture and reagents.** Cell culture reagents were purchased from GIBCO-BRL Life Technologies (Karlsruhe, Germany) and PAA (Cölbe, Germany). Human fibrosarcoma HT1080 cells were from DSMZ (German Collection of Microorganisms and Cell Cultures; Braunschweig, Germany). Rat vascular smooth muscle cells were now genotyped using single stranded conformation polymorphism (SSCP) PCR and nucleotide sequencing of the PCR products as described previously (9, 37). The Declaration of Helsinki was observed throughout the study.

<table>
<thead>
<tr>
<th>Number</th>
<th>Diagnosis</th>
<th>Sex</th>
<th>LVEF</th>
<th>AT2 1675 Genotype</th>
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<tr>
<td>1</td>
<td>DCM</td>
<td>m</td>
<td>20%</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>CHD</td>
<td>m</td>
<td>25%</td>
<td>A</td>
</tr>
<tr>
<td>3</td>
<td>DCM/MI</td>
<td>m</td>
<td>20%</td>
<td>A</td>
</tr>
<tr>
<td>4</td>
<td>DCM</td>
<td>m</td>
<td>31%</td>
<td>A</td>
</tr>
<tr>
<td>5</td>
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<td>m</td>
<td>42%</td>
<td>G</td>
</tr>
<tr>
<td>6</td>
<td>AS/AI</td>
<td>m</td>
<td>38%</td>
<td>A</td>
</tr>
<tr>
<td>7</td>
<td>Primary pulmonary hypertension</td>
<td>m</td>
<td>50%</td>
<td>G</td>
</tr>
<tr>
<td>8</td>
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<td>f</td>
<td>20%</td>
<td>G/G</td>
</tr>
<tr>
<td>9</td>
<td>CHD</td>
<td>m</td>
<td>37%</td>
<td>A</td>
</tr>
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<td>m</td>
<td>20%</td>
<td>G</td>
</tr>
<tr>
<td>11</td>
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<td>&lt;10%</td>
<td>G/G</td>
</tr>
<tr>
<td>12</td>
<td>DCM</td>
<td>m</td>
<td>&lt;20%</td>
<td>A</td>
</tr>
</tbody>
</table>

Twelve of the 14 patients with end-stage heart failure who had undergone orthotopic heart transplantation and whose explanted hearts were analyzed for AT2 mRNA splice pattern in a previous study (15) were now genotyped. Comparison of the RT-PCR results (Fig. 2A) with the respective genotype revealed that the myocardial splice pattern was not affected by the genotype. LVEF, left ventricular ejection fraction; DCM, dilated cardiomyopathy; CHD, coronary heart disease; MI, myocardial infarction; AS, aortic stenosis; AI, aortic insufficiency.

**RESULTS**

The AT2 gene 1675 G/A polymorphism (PM) is located within a putative branch site consensus motif in the small intron 1 (Fig. 1, A and B). Therefore, we examined whether it could modulate pre-mRNA splicing qualitatively or quantitatively and/or affect the level of protein expression. The AT2 mRNA splice pattern in explanted human hearts is not modified by the AT2 PM. We first determined the AT2 genotype of patients who had undergone orthotopic heart transplantation in 1997 and 1998 in the German Heart Institute and whose explanted hearts were analyzed for the AT2 splice pattern of 14 explanted failing hearts from patients who had undergone orthotopic heart transplantation at the German Heart Institute (see Table 1 for patient data). Twelve of these patients were now genotyped using single stranded conformation polymorphism (SSCP) PCR and nucleotide sequencing of the PCR products as described previously (9, 37). The Declaration of Helsinki was observed throughout the study.

**Analysis of the Effect of the AT2 1675 Genotype on the Myocardial AT2 Splice Pattern**

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**Analysis of the AT2 splice pattern in transfected cells.** One day posttransfection cells were harvested and poly A+ RNA was prepared using the Dynabeads mRNA Purification Kit (Dynal, Oslo, Norway), according to the manufacturer’s protocol. One tenth of the RNA was reverse-transcribed with Superscript II Reverse Transcriptase (Gibco Life Technologies), and 20% of the resulting cDNA was PCR-amplified in a Perkin Elmer 9600 Thermo Cycler with a primer pair binding to the cDNA of the human AT2 construct (AT2Ex1for: 5'- CAG AAT TAC AAG CAT TCT GCA GCC-3'; AT2Ex3rev: 5'- CCT AAG CTC GAA ATG TCA GCA GCT CC-3') underlined the gener-
Fig. 1. Structure of the human ANG II type 2 receptor (AT2) gene and localization of the polymorphism. A: schematic drawing based on Martin and Elton (27) of the human AT2 gene with its two introns and three exons and its mRNA splice variants (not to scale). B: localization of the 1675 G/A polymorphism (PM) (highlighted) within the small intron 1 of the AT2 gene. Open boxes mark the flanking exons 1 and 2. The shaded box marks a sequence motif with enhancer activity, which was identified in a previous study (40). Bottom: comparison between the AT2 sequence surrounding the PM and the splice branch site consensus. Note that the PM localizes to the nucleotide position 5' adjacent to the highly conserved A residue at which the lariat-splicing intermediate is formed.

Fig. 2. Myocardial AT2 mRNA splice pattern of explanted human hearts and corresponding AT2 genotypes. A: example of a RT-PCR analysis demonstrating the AT2 splice variants exon 1/2/3 (upper band) and exon 1/3 (lower band) in myocardial tissue samples from explanted human hearts [reproduced with permission from (44)]. For the present study the genotypes of the patients were determined (for a summary, see Table 1). Note that the splice pattern is identical for both genotypes. A, adenine; G guanine. In samples with lower total AT2 mRNA expression, the exon 1/3 variant approaches the detection limit. HF, heart failure; co, control; LV, left ventricle; RV, right ventricle; RA, right atrium; PDH co, pyruvic dehydrogenase control, M, 100 bp DNA size marker. B: example of a single-strand conformational polymorphism PCR analysis for the determination of the AT2 1675 PM. Arrows indicate the bands, which allow differentiation of the two alleles.
Fig. 3. AT2 mRNA splice variants in cells transfected with AT2/minigene luciferase constructs expressing either AT2 allele. A: schematic drawing of the AT2 minigene/luciferase reporter constructs used for transfection experiments. Because the constructs contain the complete transcribed, but not translated, AT2 sequence up to the translation start codon in exon 3, mRNA splicing and the postulated regulation of splicing by the PM should be identical to the endogenous AT2 alleles; B and C: results of RT-PCRs demonstrating identical AT2 mRNA splice patterns in HT1080 cells (B) and PC12W (C) cells that were transiently transfected with the minigene constructs and control PCRs for /H9252 actin. RT/H11001, reverse transcription before PCR; RT, no reverse transcription before PCR; M, 100 bp DNA size marker; co, untransfected cells; H2O, control without cDNA; plasm co, control PCR amplification of plasmids that contain the already spliced cDNA sequences (1/3 = exon 1/3, 1/2/3 = exon 1/2/3). The letters A and G indicate the allele of the AT2 minigene construct used for transfection. /H9252 Actin PCR served as a control for RNA input and quality. 32 c., 26 c. and 30 c. indicate the number of PCR cycles. Note that the AT2 primer pair used in this assay is different from that used in Fig. 2A resulting in different PCR product sizes.
and whose explanted hearts had been analyzed for the myocardial AT1 and AT2 splice pattern in a previous study (44). In this RT-PCR-based study, we found identical AT2 splice patterns in all patients, irrespective of their clinical diagnosis, stage of disease, sex or cardiac localization of the sample (i.e., atrium or ventricle, Fig. 2). In all patients the splice variant exon 1/2/3 was the most abundant AT2 mRNA (92%) compared with the small splice variant exon 1/3 (8%) (44). The SD was low for the highly abundant splice variant Ex 1/2/3 (92 ± 8%) but were high for the low-abundance variant Ex 1/3 (8+/−12%). However, the differences between expression of both mRNAs were highly significant. Importantly, irrespective of the genotype, the splice variant Ex 1/2/3 could be detected in all samples tested, and the smaller transcript Ex 1/3 was only close to the detection limit when total AT2 mRNA expression was low. For the present study, 12 of these patients (10 males, 2 females) were genotyped. Seven males were hemizygous for the AT2 A allele and 3 for the G allele; the two female patients were homozygous for the G allele (Table 1). These ex vivo data demonstrate that the myocardial AT2 mRNA splice pattern is not detectably affected by the 1675 genotype.

The AT2 splice pattern is not affected by the PM in cells transiently transfected with minigene constructs of the two alleles. Because there is no human cell line expressing the AT2 endogenously and primary cells such as vascular endothelial cells downregulate it rapidly in cell culture (2), AT2 minigene constructs were used to investigate the effect of the PM in cell cultures. The minigene constructs contained a genomic AT2 fragment of either allele comprising the core promoter, exon 1 and 2, intron 1 and 2, and exon 3 up to the translation start codon upstream of the luciferase coding region (Fig. 3A). These plasmids were transiently transfected in human HT1080, rat PC12W, and rat vascular smooth muscle cells. RT PCR analysis of the overexpressed chimeric human AT2/luciferase mRNA revealed that all three cell lines expressed the same splice pattern, irrespective of the AT2 genotype, and this pattern was identical to the previously identified myocardial AT2 splice pattern, that is, mRNA exon 1/2/3 was the predominant variant in HT1080 and PC12W cells (Fig. 3, B and C, respectively), as well as in rat vascular smooth muscle cells (not shown). Plasmids carrying the spliced AT2 cDNAs consisting of exon 1/2/3 and exon 1/3 served as positive controls (Fig. 3, B and C). AT2 signals were only observed after reverse transcription of the RNA, indicating that the signals were not due to DNA contamination. As expected, untransfected cells did not exhibit AT2 signals but were positive in the β-actin control. This confirmed the specificity of the AT2 primer pair for the introduced human AT2 transgene.

The G allele of the AT2 1675 G/A PM expresses higher protein levels than the A allele. In contrast to the identical mRNA splice pattern, luciferase activities driven by the two AT2 minigene/luciferase constructs were significantly different (Fig. 4). In rat vascular smooth muscle cells, luciferase activities expressed from the G allele construct were 54.7% ± 17.0 (P < 0.005, n = 10) higher, in PC12W cells 37.7% ± 9.9 higher (P < 0.005, n = 8) and in HT1080 cells 25.8% ± 6.6 (P < 0.005, n = 10) higher than those expressed by the A allele construct (Fig. 4). Because this cell culture model, luciferase levels may be regarded as representatives of AT2 protein levels in vivo, the data imply that the 1675 G/A PM may modulate AT2 protein expression.

DISCUSSION

Two independent studies associated the human AT2 gene 1675 G/A PM with left ventricular structure and recurrent coronary ischemia (15, 37). Because the PM is located in an intronic splice branch site, it was postulated that it might affect pre-mRNA splicing and thereby AT2 receptor expression. However, this hypothesis had not been verified convincingly. Therefore, we determined the effect of the PM systematically in 1) in vitro transfection experiments and 2) ex vivo by comparison of the myocardial AT2 splice patterns with the genotypes of 12 patients with heart failure.

In spite of careful investigation by many researchers, the function of AT2 in cardiovascular cells has not yet been completely clarified. In earlier studies, we overexpressed the human AT2 receptor in porcine cardiac fibroblasts (PC12) and human umbilical vein endothelial cells. Overexpression of AT2 receptors in these cells, which did not exhibit a native AT2, led to a 6- to 10-fold higher expression of AT2 compared with endogenous AT1 receptors. Stimulation of the overexpressed AT2 receptor in fibroblasts inhibited tyrosine phosphatase activity but had no significant effect on fibrinolysis functions such as collagen synthesis, migration, or proliferation. However, inhibition of phosphotyrosine phosphatase 1b, which regulates insulin signaling was achieved by AT2 stimulation (41).

At first, however, we performed a database search to exactly locate the PM: it is positioned in the 152-bp intron 1 of the AT2 gene, 30 bp upstream of the splice acceptor site, in a sequence motif that is indeed homologous to the splice branch site consensus (Fig. 1) (12, 34). This consensus sequence is poorly defined: The only nucleotide that is strictly conserved is the A nucleotide, at which the lariat-splicing intermediate is formed (7). In contrast, the PM is 5′ adjacent to this adenosine at a position where a purin nucleotide (A or G) is conserved. Therefore, it was questionable whether the PM might affect the AT2 mRNA splice pattern. We suggested that in the case of a
complete inactivation of the splice branch site by one of the alleles skipping the small exon 2 and a splice pattern shift toward a preponderance of the small splice variant, exon 1/3 might occur. As we demonstrated previously, this would result in an increase of AT2 receptor protein expression (7).

In earlier studies, we determined the genotype distribution and allele frequency of the AT2 gene in controls and patients with heart disease. No sex difference in gene expression was found (10). However, in myocardial tissue samples (atria and ventricles) of 12 explanted hearts from genotyped patients (7 A, 5 G allele carriers), in all three cell types transfected with the human AT2 constructs, as well as in myometrial samples, which were used as a source of AT2 mRNA in our previous work (data not shown), we identified the same pattern of AT2 mRNA expression: the mRNA consisting of exon 1/2/3 was the most abundant variant, whereas the transcript exon 1/3 was rare and sometimes below the detection limit, when total AT2 mRNA expression was low.

These data are in contrast to a study by Nishimura and colleagues (30), who identified in primary fibroblasts derived from male, and therefore hemizygous individuals, and in uterine tissue samples from homozygous women AT2 genotype-dependent differences in the AT2 splice pattern. In this study, the A allele carriers expressed the splice variant exon 1/2/3 only and at a high expression level, whereas the G allele carriers expressed splice variant exon 1/3 only and at a low level. The authors concluded—but did not confirm by protein expression data—that the G allele may be associated with a reduced AT2 receptor expression. They did not explain why a splice pattern shift should result in such a pronounced difference in total AT2 mRNA expression.

In contrast, in the present study luciferase expression levels from the AT2 minigene constructs implied that the G allele carriers expressed splice variant exon 1/3 only and at a high expression level, whereas the G allele carriers expressed splice variant exon 1/3 only and at a low level. The authors concluded—but did not confirm by protein expression data—that the G allele may be associated with a reduced AT2 receptor expression. They did not explain why a splice pattern shift should result in such a pronounced difference in total AT2 mRNA expression.

Nishimura and colleagues (30) found a strong association of the G allele with two forms (ureteropelvic junction stenosis or atresia and multicystic dysplastic kidneys) of congenital anomalies of the kidney and urinary tract (CAKUT) within two cohorts of male American Caucasians (13 patients/31 controls) and male German Caucasians (23 patients/24 controls) (35). However, this association was not confirmed in a cohort of 66 Japanese boys with CAKUT (16). Modifier genes were suggested to contribute to the incomplete penetrance of this rare disease and may explain why the association was not identified in other study populations.

In one of the studies demonstrating an association of the AT2 PM with left ventricular wall thickness, this effect was only detectable in young male individuals with mild hypertension (37). This may suggest that AT2 effects only become relevant in pathological situations, presumably because the receptor is up-regulated in the remodeling cardiovascular system (20, 42, 46) or on a certain, not yet defined, genetic background, which includes the possibility that the PM is in linkage disequilibrium with another not yet identified PM. The latter may be supported by the observation that some inbred AT2 knockout mouse strains tend to develop CAKUT (30), but most do not (14, 19).

In conclusion, our data demonstrate that the AT2 1675 PM is not associated with a qualitative modification of AT2 pre-mRNA splicing, that is, a splice pattern shift, but with different AT2 protein expression levels. Although the precise mechanism underlying this difference remains to be determined, we found that it was not related to the promoter-like activity of intron 1, which was identified in a previous work (37) (data not shown). Rather, it is conceivable that a slightly higher affinity of the splicing factors to the G allele may result in a more efficient pre-mRNA processing and thereby protein expression (13). The moderately higher AT2 expression, which may occur in the myocardium and the vasculature of G allele carriers, particularly under pathological conditions associated with an increased AT2 expression, may exert protective effects and reduce cardiac hypertrophy and coronary artery remodeling.

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