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Genetic Deletion of AT2 Receptor Antagonizes Angiotensin II-Induced Apoptosis in Fibroblasts of the Mouse Embryo

Wenge Li,* Yizhou Ye,* Bo Fu,* Jianzhong Wang,* Lifang Yu,* Toshihiro Ichiki,† Tadashi Inagami,† Iekuni Ichikawa,‡ and Xiangmei Chen*,[1]

**Division of Nephrology, General Hospital of Chinese PLA, Beijing 100853, China; and †Department of Biochemistry and ‡Department of Pediatrics, Vanderbilt University School of Medicine, Nashville, Tennessee 37232*

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To examine whether angiotensin II (Ang II) can trigger apoptosis via Ang II type 2 (AT2) receptor, two genotypes of skin fibroblasts cultured from the AT2 receptor gene targeting homozygous (AT2−/−) and wild-type (AT2+/+) mouse embryos, respectively, were studied when exposed to Ang II. In the AT2+/+ fibroblasts, mRNA expression of the AT2 receptor was modulated by Ang II in a dose-dependent manner and apoptosis appeared with the convincing features of internucleosomal DNA fragmentation and DNA content decrease after stimulation with Ang II, whereas Ang II had no significant impact on the AT2−/− fibroblasts due to the AT2 receptor gene deletion. This is the first report using a gene targeting study to demonstrate that Ang II induces apoptosis through the AT2 receptor in the fibroblasts of the mouse embryo. © 1998 Academic Press

Angiotensin II (Ang II) has long been known to play important roles in cellular hyperplasia and hypertrophy and extracellular matrix accumulation, which directly implicates organ sclerosis. There are two major Ang II receptor isoforms, AT1 and AT2. The AT1 receptor mediates the well-known pressor and mitogenic effects of Ang II, but the signaling mechanism and physiological function of the AT2 receptor remains uncertain largely. Ichiki *et al.* (1) and Hein *et al.* (2) reported that the targeted disruption of the mouse AT2 receptor gene resulted in a significant increase of both blood pressure and the sensitivity to the pressor action of Ang II. More recently, several *in vitro* studies have shown that the AT2 receptor can inhibit cell proliferation (3–8) and mediate programmed cell death (9, 10), however, these discoveries of the AT2 receptor function need vigorous examination with various cells and tissues under a variety of conditions.

The AT2 receptor gene null mice provide a new material for uncovering potential actions of the AT2 receptor. The recent reports of Yamada *et al.* and Horiuchi *et al.* (9, 10) showed that Ang II could mediate apoptosis through the AT2 receptor in PC12W and R3T3 cell lines. According to the function recognized to be induced by Ang II via the AT2 receptor, we speculate that the AT2 receptor gene deficiency may antagonize cell apoptosis when exposed to Ang II.

In both rodents and humans, the AT2 receptor gene is markedly activated in the embryonic stage and is precipitously decreased after birth, though it remains at finite and detectable levels in a few tissues (11, 12). To examine whether Ang II can induce apoptosis via the AT2 receptor as found in the PC12W and R3T3 cells, we cultured skin fibroblasts from the AT2+/+ and the AT2−/− mouse embryo on the 16th day (21 days = full term) of postcoitum (P.C.), respectively, and studied the actions of Ang II on the two genotype of the fibroblasts. The rationale of using the fibroblasts for the present study is that they are characteristically similar to the R3T3 cells as cited above.

MATERIALS AND METHODS

AT2 receptor knock out mice. Male and female homozygous mice of AT2 receptor gene null mutation (depicted as AT2−/−) and wild-type mice (depicted as AT2+/+), were prepared by Ichiki *et al.* as reported (1).

Preparing mouse embryonic fibroblasts. The skin fibroblasts were cultured from the AT2 receptor gene targeting homozygous and wild-type mouse embryos on the 16th day P.C., respectively, as described previously (13). Briefly, the mouse embryos were dissected out under ether anesthesia, limbs and head were removed, internal organs

[1] To whom correspondence should be addressed.

Abbreviations used: Ang II, angiotensin II; AT1, angiotensin II type 1 (receptor); AT2, angiotensin II type 2 (receptor); P.C., postcoitum; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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were scooped out. Embryonic tissues were minced with a sterile razor blade until they were of the consistency of sludge. The minced tissues were placed into a 15-ml screw-cap tube containing 10 ml of trypsin/EDTA in PBS and shaken in a 37°C water bath for 10 min. A 5-ml aliquot was placed in a 50-ml sterile tube with an equal volume of Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum (FCS). Five milliliters of trypsin/EDTA was added to the remaining 5 ml aliquot in the original tube and incubated at 37°C for an additional 10 min period. The second 5-ml aliquot was added to the 50-ml screw-cap tube with an equal volume of DMEM plus 10% FCS. These steps were repeated five times, only insoluble cartilage, etc., should be left in the 50-ml tubes. The contents of 50-ml tubes were centrifuged and resuspend in DMEM medium with 10% FCS, the cells were seeded into 10-cm sterile plastic tissue culture dishes. The fibroblasts of both AT +/+ and AT2 −/− were identified by the immunofluorescence stains of anti-α-smooth muscle actin and vimentin. The purity of both the AT2+/+ and the AT−/− fibroblasts was more than 98%. The third passages of the two genotypes of the fibroblasts were used in the subsequent studies.

Northern blot analysis for the AT1 and the AT2 receptor mRNA. Total RNA was isolated from the AT2+/+ fibroblasts after Ang II stimulation for 72 h at 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} mol/L, respectively, using RNAZolB (Tel Test Inc., Friendswood, TX). Ten micrograms of total RNA was electrophoresed in 1.0% agarose gel, transferred to a nylon membrane (Zetabind, Cuno Inc., Meriden, CT) and hybridized with ^{32}P-labeled AT1 and the AT2 cDNA probes (kindly presented by Dr. T. Inagami, Vanderbilt university school of medicine, Nashville TN), respectively. The membrane was rehybridized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe (Clontech, Palo Alto, CA) as a control for RNA loading. The results of Northern blot analysis for AT1 and AT2 mRNA were quantified using a computer scanning system (UVP Inc., UK).

Induction of apoptosis and its identification. 1×10^6 fibroblasts of both the AT2+/+ and the AT2−/− in the confluent state were induced by 1% FCS DMEM medium containing 10^{-7}, 10^{-6}, and 10^{-5} mol/L of Ang II, respectively, in the 75 cm^2 sterile flasks. To study the effect of blocking the AT1 and the AT2 receptor on the Ang II-induced apoptosis in the fibroblasts, losartan (Merck, West point, PA), an AT1 receptor antagonist, and PD123319 (Research Biochemicals Inc., Natick, MA), an AT2 receptor antagonist, were respectively used in the experimental condition. The fibroblasts that detached from the flasks were collected at the incubation time points of 24, 48, and 72 h. The genomic DNA of the two genotypes of the fibroblasts were extracted with phenol:chlorololoform (1:1) and chloroform. For analysis of genomic DNA fragmentation, DNA (10 μg/lane) was fractionated by electrophoresis in 1.2% agarose gel containing 2 μg/ml ethidium bromide and visualized on a UV Illuminator (UVP Inc., UK). The apoptotic fibroblasts attached to the flasks were identified by an *in situ* cell death detection kit through detection of enzymatic labeled DNA strand breaks in fixed cells (Boehringer Mannheim, Germany).

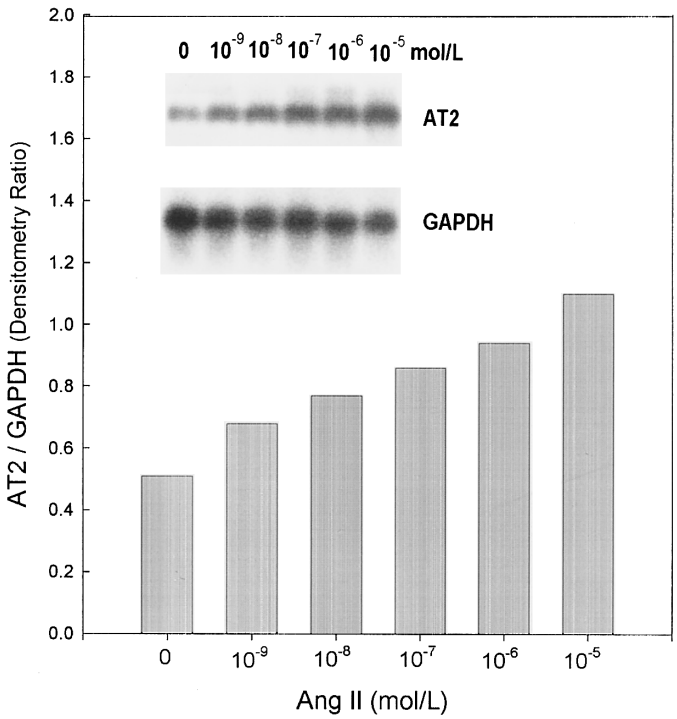

FIG. 1. Expression of the AT2 receptor in the AT2+/+ mouse fibroblasts treated with Ang II for 72 h by Northern blot analysis.

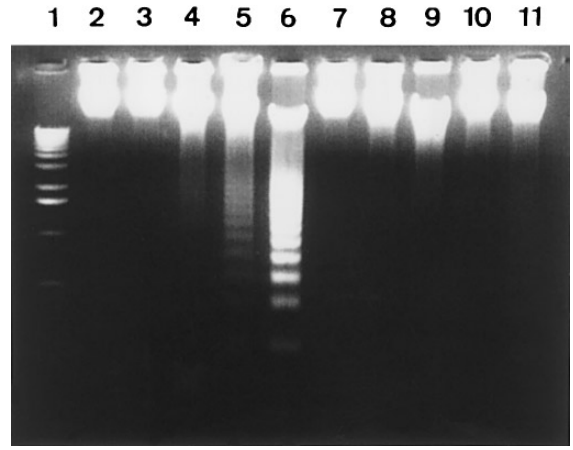

FIG. 2. Electrophoresis of DNA from the AT2+/+ (lanes 2–6) and the AT2−/− (lanes 7–11) fibroblasts having been induced by Ang II for 72 h. Molecular weight standard of 1000-bp DNA ladder was shown in Lane 1; lanes 2 and 7 for DNA of the fibroblasts cultured with 10% FCS DMEM; lanes 3 and 8, cultured with 1% FCS DMEM; lanes 4 and 9, treated with 1% FCS DMEM containing Ang II of 10^{-7} mol/L; lanes 5 and 10, treated with 1% FCS DMEM containing Ang II of 10^{-6} mol/L; lanes 6 and 11, treated with 1% FCS DMEM containing Ang II of 10^{-5} mol/L.

Flow cytometry assay. The fibroblast DNA content was assessed by flow cytometry. The AT2 +/+ and the AT2 −/− fibroblasts were collected after stimulation with Ang II for 72 h, adjusted to 1×10^6 cells /ml in PBS, centrifuged at 800 rpm for 5 minutes, fixed with 70% ethanol overnight, then centrifuged and resuspended in PBS containing 50 μg/ml of propidium iodide and 10 μg/ml of Rnase A, incubated at 37°C for 30 min, then analyzed on FACS Calibur (Becton Dickinson, USA).

RESULTS

Expression of the AT1 and the AT2 mRNA. Induced by Ang II for 72 h at the concentrations of 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} mol/L, respectively, the mRNA expression of the AT2 receptor was examined by Northern blot analysis in the AT2+/+ fibroblasts. The results showed that Ang II up-regulated the AT2 receptor gene expression in a dose-dependent pattern (Fig. 1), while in both the AT2+/+ and the AT2−/− fibroblasts, we

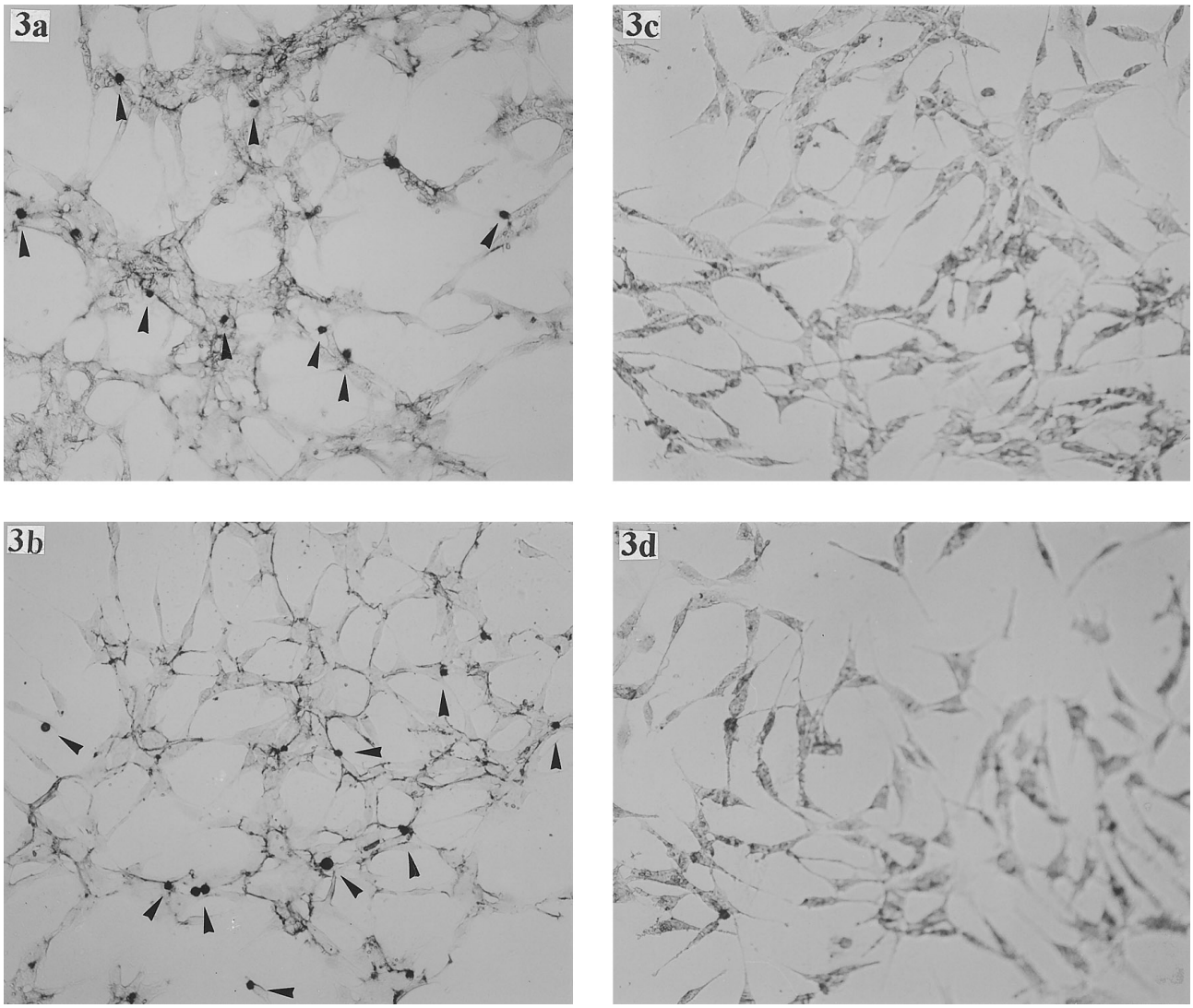

FIG. 3. Apoptotic fibroblasts (shown by arrow) identified by an *in situ* detection of enzymatic labeled DNA strand breaks among the AT2+/+ and the AT2−/− fibroblasts having been treated with Ang II for 72 h. (a) and (b) represent the AT2+/+ fibroblasts having been treated with Ang II at 10^{-6} and 10^{-5} mol/L, respectively; (c) and (d) represent the AT2−/− fibroblasts having been treated with Ang II at 10^{-6} and 10^{-5} mol/L, respectively.

failed to detect the AT1 mRNA expression by Northern blot analysis, which may implicate that the AT1 receptor was not or poorly expressed in the mouse fibroblasts.

Apoptosis assay. Genomic DNA of the fibroblasts collected at the Ang II incubation time points of 24, 48, and 72 h, respectively, was electrophoresed in 1.2% agarose gel. The genomic DNA of the AT2 +/+ fibroblasts clearly displayed a regularly spaced " ladder pattern " of internucleosomal DNA fragmentation when exposed to Ang II for 72 h at both 10^{-6} and 10^{-5} mol/L, but in the AT2 −/− fibroblast, no similarity appeared during the Ang II-induced periods at 10^{-7}, 10^{-6}, and 10^{-5} mol/L (Fig. 2). Blocking the AT2 receptor of the AT2+/+ fibroblasts by PD123319 effectively antagonized the Ang II-induced apoptosis, similar to the result found in the AT2−/− fibroblasts. losartan (an AT1 receptor antagonist) had little impact on the Ang II-mediated genomic DNA fragmentation in the AT2+/+ fibroblasts. Also, the *in situ* cell death detection demonstrated the presence of apoptotic fibroblasts in the Ang II-treated AT2+/+ fibroblasts that attached to the flasks as well as the absence of apoptotic fibroblasts in the AT2−/− fibroblasts (Figs. 3a–3d).

Flow cytometry analysis. In contrast to the AT2−/− fibroblasts, flow cytometry analysis demonstrated the presence of hypodiploid DNA peaks (A_0) among the AT2 +/+ fibroblasts that comprised the apoptotic fibroblasts which had lost some DNAs due to either fragmentation or decreased chromatin after stimulation by Ang II at 10^{-6} and 10^{-5} mol/L (Figs. 4a–4h).

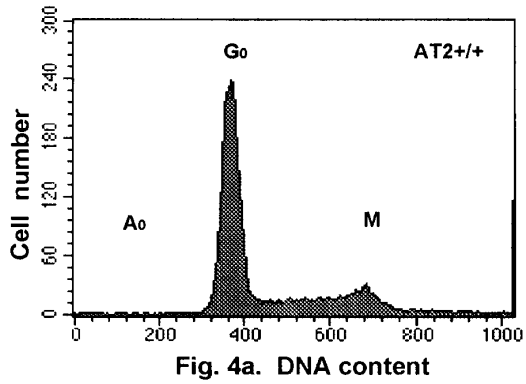

Fig. 4a. DNA content

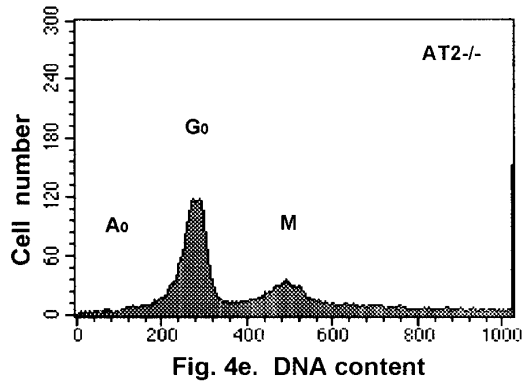

Fig. 4e. DNA content

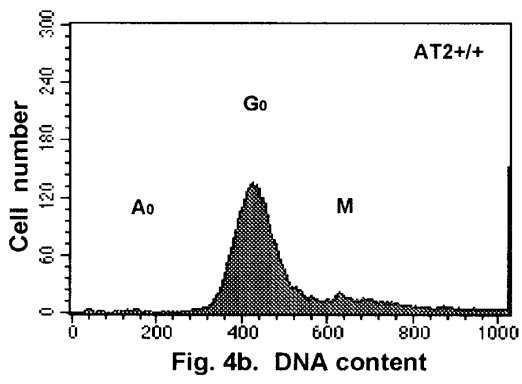

Fig. 4b. DNA content

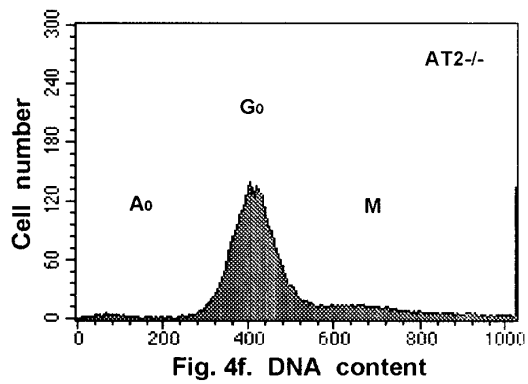

Fig. 4f. DNA content

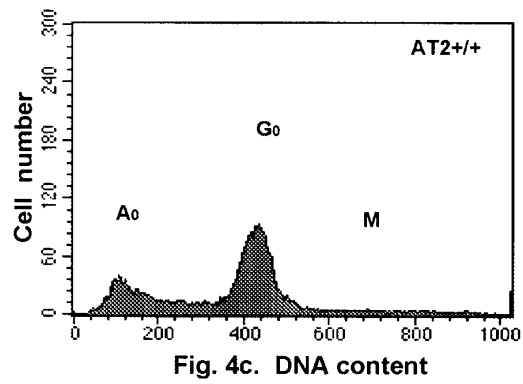

Fig. 4c. DNA content

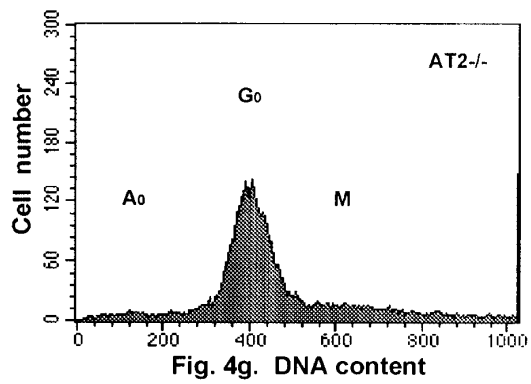

Fig. 4g. DNA content

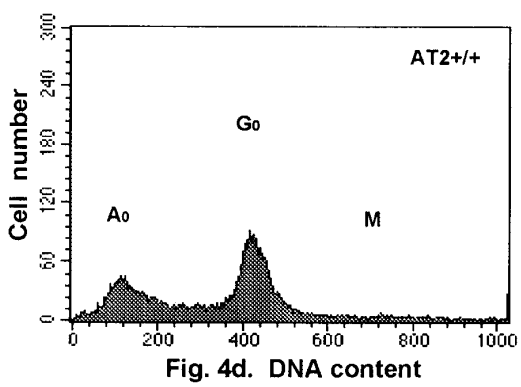

Fig. 4d. DNA content

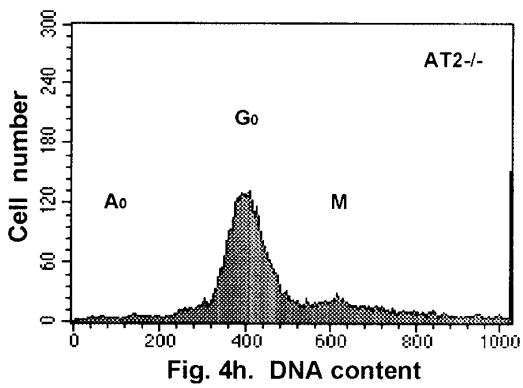

Fig. 4h. DNA content

FIG. 4. Flow cytometry analysis of apoptosis in the AT2+/+ (a-d) and the AT2−/− (e-h) fibroblasts having been treated with Ang II for 72 h. (a) and (e) for the fibroblasts cultured with 10% FCS DMEM; (b) and (f), cultured with 1% FCS DMEM; (c) and (g), treated with 1% FCS DMEM containing Ang II) of 10^{-6} mol/L; (d) and (h), treated with 1% FCS DMEM containing Ang II of 10^{-5} mol/L.

DISCUSSION

Functional interactions between the AT1 and the AT2 receptor subtypes have been a spotlight in current studies of renin-angiotensin system (14–18). However, most of the recent studies just exhibit their contrary effect on cell growth, other opposing actions of these two receptor subtypes have not been addressed. It is well known that cell growth, proliferation, or both is mediated by the AT1 receptor, while some recent *in vitro* evidences have been obtained indicating that activation of the AT2 receptor leads to an inhibition of cell proliferation possibly by inducing cell apoptosis (3–10). Under pathophysiologic conditions, the AT2 receptor could be up-regulated to control excessive cell growth mediated in part by the AT1 receptor (16–18). Thus, characterization of the angiotensin receptor subtypes will advance our knowledge of various functions of Ang II in the pathogenesis of hypertension and related diseases.

To examine whether Ang II can induce apoptosis through the AT2 receptor in other cells as well as in the PC12W and R3T3 cells, we cultured the fibroblasts from the AT2+/+ and the AT2−/− mouse embryo (in the stage the AT2 receptor gene are highly expressed as cited above). First, we examined the mRNA expression of the AT2 receptor and its regulation by Ang II in the AT2+/+ fibroblasts. We found that the AT2+/+ fibroblasts in the confluent state highly expressed the AT2 receptor, and its expression of the AT2 receptor was modulated by Ang II in a dose-dependent manner. On the other hand, we failed to detect the AT1 receptor mRNA by Northern blot analysis in both the AT2+/+ and the AT2−/− fibroblasts, suggesting that in the mouse fibroblasts the AT1 receptor is not or very lowly expressed. Thereafter, we examined whether Ang II could trigger apoptosis via the AT2 receptor in the mouse fibroblasts. According to the levels of the AT2 receptor expression in the AT2+/+ fibroblasts stimulated with Ang II, the AT2+/+ and the AT2−/− fibroblasts were exposed to Ang II at the concentration of 10^{-7}, 10^{-6}, and 10^{-5} mol/L for 24, 48, and 72 h, respectively, and the action of Ang II on programmed fibroblast death were examined by DNA gel electrophoresis, detection of enzymatic labeled DNA strand breaks, and flow cytometry analysis. Apoptosis occurred clearly in the AT2+/+ fibroblasts when exposed to Ang II for 72 h at 10^{-6} and 10^{-5} mol/L, on the contrary, Ang II had little impact on the AT2−/− fibroblasts due to the AT2 receptor gene deletion. Our results indicate that Ang II can induce apoptosis in the mouse fibroblasts through the AT2 receptor.

In summary, this is the first report using a gene targeting study to demonstrate that the AT2 receptor mediates apoptosis in the fibroblasts cultured from the mouse embryo, which further proves and advances the previous finding for the role of the AT2 receptor in the PC12W and R3T3 cell lines.

ACKNOWLEDGMENTS

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