Interferon Regulatory Factor-1 Up-regulates Angiotensin II Type 2 Receptor and Induces Apoptosis*

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The expression of the angiotensin II type 2 (AT2) receptor is developmentally and growth regulated. In cultured R3T3 cells, expression of this receptor is markedly induced at the confluent state and with serum deprivation. In this study we demonstrated that the removal of serum from culture media resulted in the induction of apoptosis in these cells and the addition of angiotensin II further enhanced apoptosis. We have previously identified an interferon regulatory factor (IRF) binding motif in the mouse AT2 receptor gene promoter region. In this report, we observed that serum removal increased IRF-1 expression, with a rapid and transient decrease of IRF-2. To prove that the changes in IRFs after serum removal mediated apoptosis and up-regulated AT2 receptor, we transfected antisense oligonucleotides for IRF-1 or IRF-2 into R3T3 cells and observed that IRF-1 antisense oligonucleotide attenuated apoptosis and abolished the up-regulation of AT2 receptor. IRF-2 antisense oligonucleotide pretreatment did not affect the onset of apoptosis after serum removal; instead, it increased AT2 receptor binding and enhanced angiotensin II-mediated apoptosis. Taken together, these results suggest that increased IRF-1 after serum starvation contributes to the induction of apoptosis and that increased IRF-1 up-regulates the AT2 receptor expression after serum starvation, resulting in enhanced angiotensin II-mediated apoptosis.

Angiotensin II (Ang II)1 exerts various actions in its diverse target tissues controlling vascular tone, hormone secretion, tissue growth, and neuronal activities. Most of the known effects of Ang II in adult tissues are mediated by the Ang II type 1 (AT1) receptor. Recently, a second receptor subtype known as AT2 receptor, we transfected antisense oligonucleotides for IRF-1 or IRF-2 into R3T3 cells and observed that IRF-1 antisense oligonucleotide attenuated apoptosis and abolished the up-regulation of AT2 receptor. IRF-2 antisense oligonucleotide pretreatment did not affect the onset of apoptosis after serum removal; instead, it increased AT2 receptor binding and enhanced angiotensin II-mediated apoptosis. Taken together, these results suggest that increased IRF-1 after serum starvation contributes to the induction of apoptosis and that increased IRF-1 up-regulates the AT2 receptor expression after serum starvation, resulting in enhanced angiotensin II-mediated apoptosis.

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1 The abbreviations used are: Ang II, angiotensin II; AT1 receptor, angiotensin II type 1 receptor; AT2 receptor, angiotensin II type 2 receptor; IRF, interferon regulatory factor; PCR, polymerase chain reaction; kb, kilobase pairs; iNOS, inducible nitric oxide synthase; EF, embryonic fibroblast; IRF-E, IRF-binding DNA element.
serum. Cells were seeded at $7 \times 10^4$ cells/T-75 flask (Falcon) for transfection experiments for internucleosomal DNA fragmentation (DNA laddering), and cells were seeded on a 24-well plate (surface area, 2 cm$^2$) (Falcon) at $2.5 \times 10^4$ cells/well for receptor binding studies. Two days after reaching confluent state, the cells were used for experiments. Cell number was counted by Coulter counter.

Oligonucleotides and Transfection to R3T3 Cells—Oligonucleotides used for treating cultured R3T3 cells as “decoy” include the following: IRF binding, $5'$-GAAAAAGAGAAGAAAATCTGTAAAAAGAT-3' mutant IRF binding, $5'$-GAAAAAGGAAAAAGAAAATCTGCTGATTTAAAGGATA-3' and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control to standardize the amount of total RNA actually blotted onto the membrane. In mutant IRF-binding oligonucleotide, three repeats of putative IRF binding hexamer motif as previously reported (15) were replaced with C4 oligomer (AAAGGA), which does not bind to IRFs (16). Oligonucleotides were annealed to complementary sequences and used as double-stranded oligonucleotides.

Phosphorothioate oligonucleotides for treating cells as antisense DNA for IRF-1 and IRF-2 are as follows: IRF-1 antisense, $5'$-GATGCCCGAGATGC-3' and $3'$-GATGCCCGAGATGC-5' and IRF-2 antisense, $5'$-GTGGAGTGGTGGTTAGGG-3' and $3'$-GAGTGTTGTTAGGG-5'. For IRF-2, PCR primers were $5'$-CTTCATCTCCGGTTGAAGACATG-3' and $5'$-CTTCATCTCCGGTTGAAGACATG-5'. In mutant IRF-binding oligonucleotide, three repeats of putative IRF binding hexamer motif as previously reported (15) were replaced with C4 oligomer (AAAGGA), which does not bind to IRFs (16). Oligonucleotides were annealed to complementary sequences and used as double-stranded oligonucleotides.

Preparation of cDNAs for IRF-1 and IRF-2 by Reverse Transcription-PCR—Total RNA was prepared from cultured R3T3 cells using RNAzol (Tel-Test), reverse transcribed using reverse transcriptase and random hexamers (Perkin-Elmer), and applied to PCR. PCR primers for IRF-1 used were as follows: 5'-CTGATGACCAGACAGTTAC-3' and 5'-CTTCATCTCCGGTTGAAGACATG-3'. For IRF-2, PCR primers were 5'-GCGGCTCTGAGCTACCTGATA-3' and 5'-CTCCCTTCGATGACCTGACCCGG-3'. PCR reaction was carried out with 30 cycles of 1 min f denaturation at 94 °C, 1 min of annealing at 55 °C, and 3 min of extension at 72 °C, followed by 15 min of final extension step. PCR-amplified DNAs were subcloned into pCR™III Vector (Invitrogen). cDNA probes for Northern blotting were prepared from these plasmid vector by restriction enzyme cut by EcoRI.

Northern Blot Analysis—Total RNA was prepared from cultured R3T3 cells using RNAzol (Tel-Test) $24$ h after oligonucleotide treatment. RNA (20 μg/lane) was separated by electrophoresis and transferred onto a nylon membrane (Amersham), and hybridization was carried out using a $^{32}$P-labeled IRF-1 and IRF-2 cDNA, a $^{32}$P-labeled HindIII-NsiI fragment of mouse AT$_2$ receptor, receptor cDNA (25), or 0.78-kb NsiI-HinDIII fragment of a human glyceraldehyde-3-phosphate dehydrogenase clone. Densitometric analysis of autoradiograms was performed by scanning densitometer (GS300, Hoeffer) and NIH Image software.

Immunoblot Analysis—R3T3 cells were seeded onto 10-cm dishes (Falcon) at $2 \times 10^5$ cells/dish for this experiment. The cells were washed with HEPES-buffered saline and lysed in 0.5 ml of phosphate-buffered saline containing $1\%$ Nonidet P-40, $0.5\%$ sodium deoxycholate, $0.1\%$ SDS, $1$ mM phenylmethylsulfonyl fluoride, $1$ mM sodium orthovanadate, and $10$ μg/ml aprotinin. Cell lysates were clarified by centrifugation at 8,500 × g for 30 min, boiled in Laemmli loading buffer for 3 min, resolved by 12% SDS-polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose membrane, and immunoblotted with IRF-1 or IRF-2 antibody (Santa Cruz Biotechnology). Antibodies were detected by horseradish peroxidase-linked secondary antibody using the ECL (enhanced chemiluminescence) system (Amersham).

Internucleosomal DNA Fragmentation (DNA Ladder)—DNA extraction, subsequent 3’ end-labeling of DNA, gel electrophoresis, and quantitation of DNA fragmentation were performed as described previously (28). Briefly, 500 ng of DNA prepared from each treated cells was

**Fig. 1.** IRF-1 and IRF-2 mRNA expression after serum starvation. Total RNA was prepared from cultured confluent R3T3 cells 3, 6, 12, 24, and 48 h after serum removal. RNA (20 μg/lane) was separated by electrophoresis and hybridized sequentially with a probe for IRF-1, IRF-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control to standardize the amount of total RNA actually blotted onto the membrane. Panel A is a representative of data obtained in four different experiments (IRF-1, 1-day autoradiographic exposure; IRF-2, 2-day autoradiographic exposure). In panel B, the signal density of each RNA sample hybridized to IRF-1 and IRF-2 was divided by that hybridized glyceraldehyde-3-phosphate dehydrogenase. The corrected density for each time point is represented as percent of the value obtained before serum depletion (time 0). The values were expressed as mean ± S.D. obtained from four separate experiments. * shows $p < 0.01$ compared with time 0.
RESULTS

Role of IRF-1 and IRF-2 in AT2 Receptor: Up-regulation after Serum Growth Factor Removal—We first examined the changes of IRF-1 and IRF-2 gene expressions after serum removal which induces apoptosis in this cell line (11). As shown in Fig. 1, expression of IRF-1 increased in a time-dependent manner and showed a maximum at 24 h after serum removal, whereas IRF-2 mRNA transiently decreased 3 h after serum removal and returned to basal level at 24 h. To document that these change in IRF-1 and IRF-2 contributed to the increased AT2 receptor expression, we treated confluent R3T3 cells with these oligonucleotides spanning IRF binding sequence (Fig. 2), serum depletion enhanced AT2 receptor binding. Pre-treatment with IRF-binding decoy oligonucleotide attenuated the increase of AT2 receptor in response to serum removal. IRF-binding decoy oligonucleotide treatment did not change the AT2 receptor density in serum-fed R3T3 cells. Mutant IRF-

oligonucleotide transfection, serum was removed from the medium. The AT2 receptor binding was determined 3 days after serum removal by radioligand binding assay (C). The values were expressed as mean ± S.D. obtained from five different cell culture wells. * shows p < 0.01 compared with LipofectAMINE transfection alone.

Fig. 2. Effects of oligonucleotides spanning IRF binding sequence in mouse AT2 receptor promoter region and antisense oligonucleotides for IRF-1 and IRF-2 on AT2 receptor expression after serum starvation. Two double-stranded oligonucleotides (IRF and mutant IRF-binding decoy oligonucleotides) (see “Experimental Procedures”) were transfected into confluent R3T3 cells, and 1 day after oligonucleotides transfection, serum was removed from the medium. The AT2 receptor binding was determined 3 days after serum removal by radioligand binding assay. A, the values were expressed as mean ± S.D. obtained from five different culture wells. Total RNA was prepared from R3T3 cells 24 h after serum removal or serum-fed cells. RNA (20 μg/lane) was separated by electrophoresis and hybridization was carried with a probe for the AT2 receptor or for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. Similar results were obtained in three different experiments (B). Antisense oligonucleotides for IRF-1 and IRF-2 were transfected into R3T3 cells. One day after oligonucleotide transfection, serum was removed from the medium. The AT2 receptor binding was determined 3 days after serum removal by radioligand binding assay (C). The values were expressed as mean ± S.D. obtained from five different culture wells. * shows p < 0.01 compared with LipofectAMINE transfection alone.
binding decoy oligonucleotide treatment did not affect AT\textsubscript{2} receptor density. We also measured AT\textsubscript{2} receptor mRNA levels at 24 h after serum removal and observed that pretreatment with IRF-binding decoy oligonucleotide inhibited the AT\textsubscript{2} receptor mRNA increase (Fig. 2B), supporting the contention that the change in the ratio of IRF-1/IRF-2 after serum depletion is important for the transcriptional up-regulation of AT\textsubscript{2} receptor expression.

To prove that the changes of IRF-1 and IRF-2 expression after serum depletion regulates the AT\textsubscript{2} receptor expression, we transfected antisense oligonucleotides for IRF-1 or IRF-2 into R3T3 cells 24 h before serum removal. To confirm whether these antisense oligonucleotides inhibit IRFs expression, we examined the IRFs protein expression 12 h after serum depletion. As shown in Fig. 3, consistent with the mRNA expression of IRF-1 (Fig. 1A), we observed that IRF-1 protein was up-regulated after serum depletion and IRF-1 antisense pretreatment inhibited increased IRF-1 expression, whereas IRF-1 sense and IRF-2 sense and antisense oligonucleotides did not influence the IRF-1 expression. On the other hand, IRF-2 protein level was not changed 12 h after serum depletion, although IRF-2 mRNA transiently decreased 3 h after serum removal and returned to basal level rapidly. IRF-2 antisense oligonucleotide treatment decreased the expression of IRF-2. Next we examined the effects of IRFs antisense oligonucleotides on AT\textsubscript{2} receptor expression and observed that IRF-2 antisense enhanced AT\textsubscript{2} receptor binding and IRF-1 antisense oligonucleotide attenuated AT\textsubscript{2} receptor binding in confluent serum-fed R3T3 cells (Fig. 2C). We demonstrated further that IRF-1 antisense oligonucleotide pretreatment abolished the up-regulation of AT\textsubscript{2} receptor expression after serum removal (Fig. 2C), demonstrating that the increase in IRF-1 mediated the up-regulation of AT\textsubscript{2} receptor expression. In the serum-depleted state, IRF-2 antisense treatment increased the AT\textsubscript{2} receptor density. These data suggest that increased IRF-1 and decreased IRF-2 after serum removal may exert synergistic effects on AT\textsubscript{2} receptor up-regulation.

**Induction of Apoptosis by IRF-1**—To test the possibility that the increased expression of IRF-1 in R3T3 cells after serum growth factor removal contributes to the induction of apoptosis, we treated the R3T3 cells with antisense oligonucleotides for IRF-1 or IRF-2 1 day before serum starvation. As shown in Fig. 4, serum depletion induced the internucleosomal cleavage of DNA, which resulted in the generation of DNA fragments of multiples of ~180 base pairs (the size of a nucleosome): a hallmark of apoptosis. IRF-1 antisense oligonucleotide treatment inhibited DNA fragmentation in serum-starved R3T3 cells, whereas IRF-1 antisense did not affect DNA fragmentation in serum-fed R3T3 cells, suggesting that the increase of
II was significantly greater in serum-starved cells. Next we serum-fed and serum-starved cells; however, the effect of Ang of DNA laddering. Representative DNA laddering is shown in A after serum growth factor removal, consistent with the results cleotide pretreatment attenuated the induction of apoptosis as shown in Fig. 5, serum depletion caused apoptotic 11956 fragment, we also examined the morphological changes of to the process of apoptosis as well. In addition to the DNA enhanced the IRF-1 effect and consequently contributed in apoptosis. On the other hand, IRF-2 antisense oligonucleotide. These studies suggest that the expression of AT2 receptor in cultured vascular smooth muscle cells was down-regulated by the addition of growth factors such as platelet-derived growth factor-BB, epidermal growth factor, and endothelin-1. Recently we have reported that this receptor induces apoptosis in R3T3 cells; however, the “genetic evidence” that AT2 receptor induces apoptosis still remains to be proved. The antigrowth effects of AT2 receptor on vascular smooth muscle cells (9) and endothelial cells (10) have been reported recently. The highly abundant expression of AT2 receptor during embryonic and neonatal growth, the rapid disappearance after birth (4, 6), and the up-regulation of AT2 receptor in myocardial infarction (27), cardiac hypertrophy (28), and skin wound (29) suggest that this receptor is closely involved with growth, development, and/or differentiation. Dudley et al. (14) reported that the addition of growth factors, such as basic fibroblast growth factor or serum, to quiescent R3T3 cells caused a rapid decrease in the number of surface AT2 receptor sites. Leung et al. (30) reported that nerve growth factor treatment decreased AT2 receptor expression in PC12W cells. Kambayashi et al. (31) also reported that the expression of AT2 receptor in cultured vascular smooth muscle cells was down-regulated by the addition of growth factors such as platelet-derived growth factor-BB, epidermal growth factor, and endothelin-1. Recently we have reported that this receptor induces apoptosis in PC12W cells by antagonizing the effect of nerve growth factor, and that apoptotic PC12W cells exhibited up-regulated AT2 receptor density (11). Moreover, we demonstrated that Ang II enhanced apoptosis in serum-starved R3T3 cells, along with the increased concentration of the AT2 receptor. These studies suggest that the expression of the AT2 receptor is inversely related to cell growth and is positively correlated with apoptosis. Consistent with our previous reports (11), we also demonstrated in this paper that AT2 receptor induces apoptosis in R3T3 cells; however, the “genetic evidence” that AT2 receptor induces apoptosis still remains to be proved.

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investigate the growth-regulated AT\textsubscript{2} receptor expression, we first cloned mouse AT\textsubscript{2} receptor genomic DNA and studied its promoter function in R3T3 cells (15). We identified IRF binding motif (IRF-Es) in the promoter region of AT\textsubscript{2} receptor gene and demonstrated that IRF-2 attenuated the AT\textsubscript{2} receptor expression in both growing and confluent R3T3 cells, whereas IRF-1 enhanced AT\textsubscript{2} receptor expression in the confluent cells only. Consistent with this result, gel mobility shift assay demonstrated that growing R3T3 cells exhibited only IRF-2 binding, whereas confluent cells exhibited both IRF-1 and IRF-2 bindings. In this paper, we demonstrated further that, in confluent R3T3 cells, the removal of serum increased IRF-1 expression but caused a rapid and transient decrease in IRF-2 mRNA expression. These changes of IRFs were associated with an up-regulation of AT\textsubscript{2} receptor and the enhancement of AT\textsubscript{2} receptor-mediated apoptosis. These results suggest that the IRFs play an important role in the up-regulation of this receptor associated with apoptosis.

IRF-1 and IRF-2 are structurally related, and they recognize the same DNA sequence elements AAGTGA motif or G(A)AAAG/CT/CGAAAG/CT/C (16, 17). Gene transfection studies have demonstrated that these two factors are mutually antagonistic; IRF-1 activates transcription, whereas IRF-2 inhibits it (16, 19). Harada et al. (23) have shown that the IRF-1/IRF-2 expression ratio oscillates throughout the cell cycle of NIH 3T3 cells, with IRF-1 expression at its highest when cells are growth-arrested owing to serum starvation and with IRF-2 being more abundant in growing cells. IRF-1 manifests anti-proliferative properties (20, 22), whereas overexpression of the repressor IRF-2 leads to cell transformation and increased tumorigenicity, and this phenotype can be reverted by the concomitant overexpression of IRF-1 (23). Accordingly, we speculate further that the changes of IRF-1 and IRF-2 after serum removal are closely linked to the induction of apoptosis in R3T3 cells. Indeed our data demonstrated an increased IRF-1 expression and transiently decreased IRF-2 expression during apoptosis induced by serum removal.

Several lines of evidence has shown that the expression of oncogenes can sensitize cells to undergo apoptosis, particularly under conditions of low serum concentration or high cell density (32, 33). It has been demonstrated that IRF-1 and IRF-2 have antioncogenic and oncogenic activities, respectively. Deletion or inactivation of the IRF-1 gene at one or both alleles have antioncogenic and oncogenic activities, respectively. Deletion or inactivation of the IRF-1 gene at one or both alleles has been detected in leukemia and myelodysplastic syndromes (34). Tanaka et al. (35) reported that IRF-1 may be a critical determinant of onogene-induced cell transformation or apoptosis in mouse embryonic fibroblasts (EFs). They demonstrated that Ras signaling, under conditions of low serum or at high cell density or following treatment by anticancer drugs or ionizing radiation, induces the death of EFs derived from wild type mice and from mice with a null mutation in the IRF-2 gene (IRF-2\textsuperscript{−/−} mice), but not of EFs from IRF-1\textsuperscript{−/−} and double knockout mice. These data demonstrate that the absence of IRF-1 alone is sufficient to prevent Ras-induced apoptosis. Tamura et al. (36) also showed that the mitogen induction of the interleukin-1β-converting enzyme, mammalian homologue of the Caenorhabditis elegans cell death gene ced-3, is IRF-1-dependent. These studies are consistent with our present results and support further that IRF-1 is a unique transcription factor that functions as an apoptotic inducer.

IRF-1 and IRF-2 are originally identified as regulators of type I interferon system. DNA sequences recognized by IRFs have been also found in the regulatory regions in a number of interferon-inducible genes (16–19). IRF binding consensus element was also identified in inducible nitric oxide synthase (iNOS) promoter region, and IRF-1 is essential for iNOS activation in murine macrophages (40). Recently we reported that NO donor molecules, S-nitroso-N-acetylpenicillamine or sodium nitroprusside, induced apoptosis in rabbit vascular smooth muscle cells (41). In this study, we observed that an increase in the ratio of IRF-1/IRF-2 after serum starvation mediated the up-regulation of AT\textsubscript{2} receptor, resulting in the enhancement of Ang II-mediated apoptosis. Our data showed that the blockade of IRF-1 expression resulted in the simultaneous reduction in AT\textsubscript{2} receptor expression and in apoptosis, whereas blockade of IRF-2 expression led to an increase in AT\textsubscript{2} receptor expression and enhanced apoptosis. Taken together these data support the notion that AT\textsubscript{2} receptor is one of the target genes of the IRFs system. We propose further that IRF-regulated gene products such AT\textsubscript{2} receptor and iNOS may play important mediating roles in IRF-regulated apoptosis.

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