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Hypoxia Induces Transcription Factor ETS-1 via the Activity of Hypoxia-Inducible Factor-1

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ETS-1 plays an important role in angiogenesis and cancer invasion, and hypoxia is a common feature in these phenomena. We examined whether hypoxia influenced ETS-1 expression. Hypoxia induced ETS-1 in a human bladder cancer cell line, T24, and promoter analysis revealed that the deletion of −424 to −279 bp from the human ETS-1 promoter decreased the hypoxia-mediated inducibility. This region contained a hypoxia responsive element-like sequence, and HIF-1 bound to it under the hypoxic condition. Double-stranded synthetic oligonucleotides of this sequence as a decoy inhibited the hypoxia-mediated induciblity. These results indicate that hypoxia induces ETS-1 via the activity of HIF-1.

Key Words: **ETS-1; hypoxia; HIF-1; angiogenesis; tumor invasion.**

The ETS family of transcription factors is defined by a conserved DNA-binding ETS domain that forms a winged helix-turn-helix structural motif. This family of transcription factors is involved in a diverse array of biological functions including cellular growth, migration, and differentiation (1). ETS-1 is the first found member of the ETS family of transcription factors. Earlier we showed that ETS-1 was induced in endothelial cells (ECs) during angiogenesis or reendothelialization after denudation (2, 3). Representative angiogenesis stimulators such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) induced ETS-1 in ECs via the activation of a classical MAP kinase, ERK1/2 (4, 5). Endothelial ETS-1 was found to be responsible for the promotion of angiogenesis (2, 6), and the elimination of the activity of ETS-1 by a dominant negative molecule inhibited angiogenesis *in vivo* (7).

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Other lines of evidence suggest that ETS-1 is expressed in various cancer cells including gastric carcinoma (8) and lung carcinoma (9). Moreover, the spatial and temporal pattern of ETS-1 expression in cancer cells suggested its role in tumor invasion. Indeed, invasion by glioma cells was blocked by the expression of mutant Ets-1 lacking its transactivation domain (10), or by antisense Ets-1 oligonucleotide (11).

Hypoxia is a common feature of solid tumors and is closely related to angiogenesis. Hypoxia activates hypoxia inducible factor 1 (HIF-1), a basic helix-loop-helix (bHLH)/Per-AHR-ARNT-Sim (PAS) transcription factor. HIF-1 is a heterodimer transcription factor consisting of a constitutively expressed ARNT/HIF-1β subunit and an O_2-regulated HIF-1α or HIF-2α subunit (12). Here, we examined whether hypoxia influenced the expression of ETS-1. Our results disclosed for the first time that hypoxia induced ETS-1 expression via the activity of HIF-1. This interaction of HIF-1 with ETS-1 would be expected to play a part in the modulation of the properties of cancer cells as well as in angiogenesis.

MATERIALS AND METHODS

Cell culture and exposure to hypoxia. Human bladder cancer cell line T24 was cultured in Dulbecco's modified Eagle medium (DMEM; Nissui Pharmaceutical Co., Ltd. Tokyo, Japan) containing 5% fetal calf serum (FCS), 4 mM glutamate, 100 μg/ml kanamycin, and 2.2 mg/ml $NaHCO_3$. Human umbilical vein endothelial cells (HUVECs) were obtained from KURABO (Osaka, Japan) and cultured on type-I collagen-coated dishes in endothelial basal medium containing endothelial cell growth supplements (Clonetics Corp., Walkersville, MD) and 5% FCS (Summit Biotechnology, Ft. Collins, CO). Cells were maintained at 37°C in a humidified incubator containing 20% O_2 and 5% CO_2 in air (referred to as the normoxic condition).

For the hypoxia experiments, cells were placed in a hypoxia chamber (Bellow Glass, Inc., Vineland, NJ) containing 0% O_2, 5% Co_2, and 95% N_2; and the hypoxia chamber was maintained at 37°C.

Northern blot analysis. Northern blot analysis for ets-1 mRNA was performed as described previously (2). Briefly, T24 cells were preincubated for 24 h in DMEM containing 0.1% bovine serum

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albumin (BSA) and were then exposed to normoxia or hypoxia. In some experiments, cells were treated with 100 μM $CoCl_2$ or incubated in the presence of 5 μg/ml actinomycin D. After the incubation, total RNA was extracted, fractionated on a 1% agarose gel containing 2.2 M formaldehyde, and transferred to a Hybond N^+ filter (Amersham Life Sciences Inc., Buckinghamshire, UK). The filter was hybridized with [^{32}P]-labeled probe in hybridization solution overnight at 42°C. The filter was thereafter washed in 2× SSC and 0.1% SDS at room temperature, and then in 0.2× SSC and 0.1% SDS at 65°C. Autoradiography was carried out with a BAS2000 Image Analyzer (Fuji, Tokyo, Japan).

Western blot analysis. Western blot analysis for ETS-1 protein was performed as described previously (2). Briefly, T24 cells were preincubated for 24 h in DMEM containing 0.1% BSA and were then exposed to hypoxia. Thereafter, the cells were extracted, and the resultant samples were separated on 10% SDS–polyacrylamide gel under reducing conditions and transferred to a nitrocellulose membrane (Hybond ECL; Amersham Life Sciences Inc.). The filter was incubated with rabbit polyclonal anti-human Ets-1 antibody (Cambridge Research Biochemicals, Cambridge, UK) for 1 h at room temperature, and subsequently incubated for 1 h with horseradish peroxidase-conjugated protein G (Bio-Rad, Hercules, CA). Immunoreactive bands were developed with an enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Life Sciences Inc.) and were visualized with a LAS-1000 Image Analyzer (Fuji).

Electrophoretic mobility shift assay. T24 cells were preincubated in DMEM containing 0.1% BSA for 24 h, and then exposed to hypoxia for 24 h. Nuclear cell extract (NCE) was prepared according to the method described by Schreiber *et al.* (13). The synthetic oligonucleotides (a normal probe, GCCTCACGTCCTGTGTGT and a mutant probe, GCCTCAAAACCTGTGTGT) were annealed and their five ends were labeled by T4 polynucleotide kinase using [γ-^{32}P] ATP (Amersham Life Sciences Inc.). The DNA probe (20,000 c.p.m.) was added to 0.15 μg of poly(dI-dC), 20 mM Hepes-KOH (pH 7.9), 100 mM KCl, 6 mM $MgCl_2$, 0.5 mM EDTA, 5% glycerol, 0.5 mM DTT, and 5 μg of NCEs in a final volume of 20 μl and incubated for 30 min at room temperature. Unlabeled probes were added to the mixture for competition. For supershift experiments, 1 μl of monoclonal antibodies against human HIF-1α or human ARNT1/HIF-1β (Transduction Laboratories, San Diego, CA) was added into the mixture, which was then incubated at 4°C for 30 min prior to the addition of DNA. The mixture was separated on a native 4.4% polyacrylamide gel in 1× TAE for 90 min. Autoradiography was carried out with an imaging plate and analyzed with a BAS2000 Image Analyzer.

Luciferase assay. Human ETS-1 promoter was kindly provided by Dr. Dennis Watson (Medical University of South Carolina) (14). We constructed ETS-1-Luc (-424 bp) and ETS-1-Luc (-279 bp). T24 cells were plated in 24-well tissue culture dishes at 5×10^4 cells/well and incubated for 2 days. By use of LipofectAMINE PLUSTM Reagent (Life Technologies, Inc., Gaithersburg, MD), subconfluent T24 cells were transfected for 3 h with 0.2 μg of luciferase constructs and pRL-TK plasmid, which contained a herpes simplex virus thymidine kinase promoter upstream of the renilla luciferase gene, and then incubated for 12 h in DMEM containing 5% FCS. The transfected cells were preincubated in DMEM containing 0.1% BSA for 24 h, and then exposed to normoxia or hypoxia for another 24 h. After the incubation, the cells were washed with PBS, and lysed with Passive lysis buffer (Promega, Madison, WI). Firefly luciferase and renilla luciferase activities were measured sequentially by using a Dual-Luciferase Reporter assay system (Promega) and a Luminescencer JNR (ATTO, Tokyo, Japan). After measuring the firefly luciferase signal (FLS) and the renilla luciferase signal (RLS), we calculated the relative luciferase activity as FLS/RLS.

Transcription factor decoy. Transcription factor decoy was applied according to the method described by Morishita *et al.* (15). T24 cells (8×10^4)/dish were plated in 6-cm plastic dishes and incubated in DMEM containing 5% FCS for 24 h. Thereafter, by use of LipofectAMINE Reagent (Life Technologies, Inc.), the cells were transfected with synthetic double-stranded oligonucleotides (0.5 μM) at 37°C for 6 h in OPTI-MEM (Life Technologies, Inc.). After the transfection, cells were incubated in DMEM containing 0.1% BSA under the normoxic or hypoxic condition for 16 h.

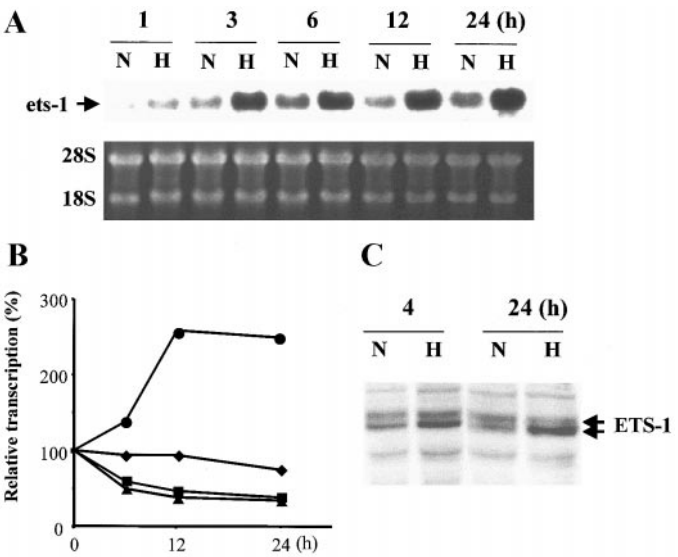
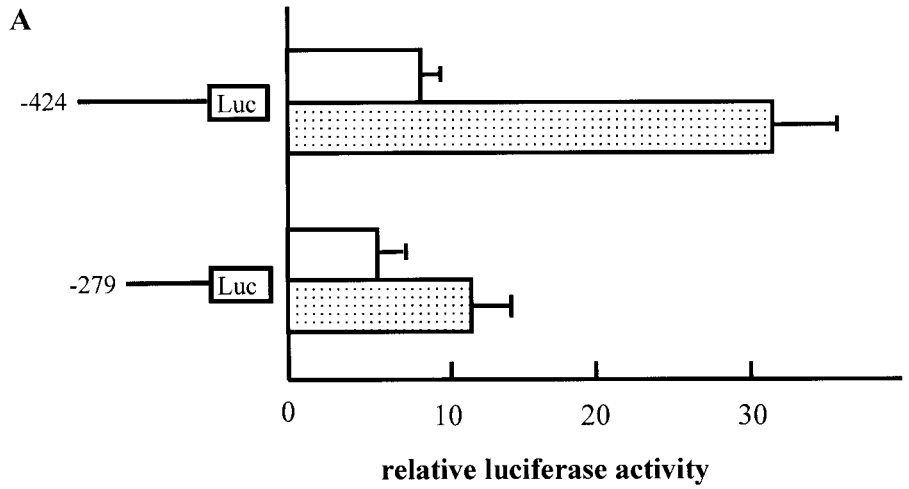


FIG. 1. Effect of hypoxia on the expression of ETS-1. (A) T24 cells were subjected to either normoxia (N) or hypoxia (H) for the indicated periods. Thereafter, the expression of ets-1 mRNA was determined by Northern blotting. (B) T24 cells were exposed to normoxia or hypoxia in the absence or presence of actinomycin D, and incubated for up to 24 h. Total RNA was extracted, and ets-1 mRNA expression was analyzed by Northern blotting. Quantification of ets-1 mRNA was performed by using BAS 2000, and the amount of ets-1 mRNA was corrected by the amount of ribosomal RNA. The basal level was defined as 100%, and values were expressed as relative expression. (●): hypoxia without actinomycin D, (◆) normoxia without actinomycin D, (■) hypoxia with actinomycin D, (▲) normoxia with actinomycin D. (C) T24 cells were incubated under either normoxia (N) or hypoxia (H) for the indicated periods. Thereafter, the expression of ETS-1 protein was determined by Western blotting.

RESULTS AND DISCUSSION

We exposed T24 cells to hypoxia, and examined the expression of ets-1 mRNA by Northern blotting. As shown in Fig. 1A, the expression of ets-1 mRNA was augmented as early as 3 h after exposure to hypoxia. Hypoxia also induced ets-1 mRNA in HUVECs (data not shown). We clarified whether hypoxia augmented ets-1 mRNA at the level of mRNA stability or transcription. Cells were exposed to normoxia or hypoxia in the absence or presence of actinomycin D, and incubated for up to 24 h. The expression of ets-1 mRNA was analyzed by Northern blotting. When cells were treated with actinomycin D, the expression of ets-1 mRNA was almost the same between normoxic and hypoxic conditions (Fig. 1B). Therefore, we concluded that hypoxia augmented ets-1 mRNA at the level of transcription. Western blotting further revealed that



B

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-420 tcctaaagcc ctttgaggac acgggctcac gaatcccctg cgcctgcctg
-370 cacgctcgcc ttcatccaca tgcctcacgt cctgtgtgtc agtctttgtg
-320 aatgaatgat gtacacgcac ttggaaaact atgctgctac tgggaggggg
-270 cgagagcggg tgaccaagcc ctcaagaatg cgtggagaat cagacggact
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FIG. 2. Promoter activity of human ets-1. (A) The luciferase activities of ETS-1-Luc (-424 bp) and ETS-1-Luc (-279 bp) were determined under normoxic (open bar) or hypoxic (stippled bar) conditions. (B) The promoter of human ets-1 is shown. Core sequence of HRE was shown in a thick character. Underlined sequence was used for the following EMSA and the decoy experiment.

the protein level of ETS-1 was increased after exposure to hypoxia as well (Fig. 1C).

Next, we examined the promoter activity of human ETS-1 by the luciferase assay. Hypoxia significantly increased the luciferase activities of ETS-1-Luc (-424 bp) (Fig. 2). These results indicated that hypoxia augmented ets-1 mRNA at the level of transcription. Interestingly, ETS-1-Luc (-279 bp) showed reduced inducibility (Fig. 2A), suggesting that a specific region of the human ETS-1 promoter between −424 and −279 was responsible for this induction. We searched the nucleotide sequence between −424 and −279 of human ETS-1 promoter, and found one consensus core sequence of hypoxia responsive element, ACGT (Fig. 2B). By using this region as a probe, we performed EMSA. EMSA revealed that a band induced by hypoxia was present (Fig. 3A, arrow). This inducible band disappeared with an unlabeled normal probe but not with a mutant probe (Fig. 3B). Moreover, this inducible band was supershifted by anti-HIF-1α antibody as well as anti-ARNT/HIF-1β antibody (Fig. 3C; asterisk). These results indicate that a heterodimer of HIF-1α and ARNT/HIF-1β bound to the specific region of human ETS-1 promoter under the hypoxic condition.

To further clarify the requirement of HIF-1 in hypoxia-mediated induction of ETS-1, we constructed a double-stranded synthetic oligonucleotide of the HRE-like sequence, the same sequence that we used for EMSA, and transfected cells with it as the transcription factor decoy. Northern blotting revealed that the transfection with the decoy almost completely inhibited the induction of ets-1 mRNA by hypoxia (Fig. 4,

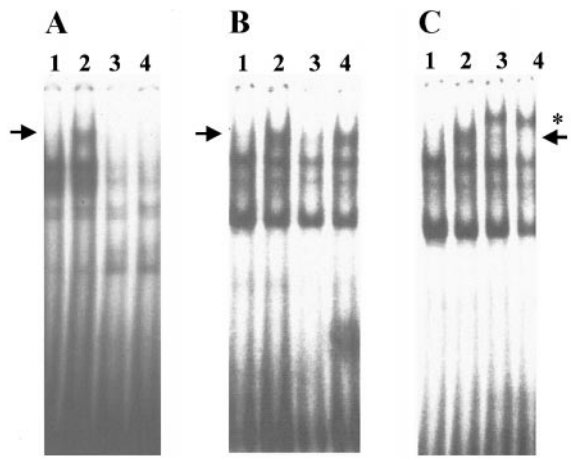

FIG. 3. Binding of HIF-1 to human ETS-1 promoter. T24 cells were exposed to hypoxia and NCE was prepared. Thereafter, EMSA was performed as described under Materials and Methods. (A) Lane 1, normoxia with a normal probe; lane 2, hypoxia with a normal probe; lane 3, normoxia with a mutant probe; lane 4, hypoxia with a mutant probe. (B) Lane 1, normoxia; lane 2, hypoxia; lane 3, hypoxia with normal competitor (50-fold over the labeled probe); lane 4, hypoxia with mutant competitor (50-fold over the labeled probe 4). (C) Lane 1, normoxia; lane 2, hypoxia; lane 3, hypoxia with anti-HIF-1α Ab; lane 4, hypoxia with anti-ARNT-1/HIF-1β Ab. Arrow, inducible band; asterisk, supershifted band.

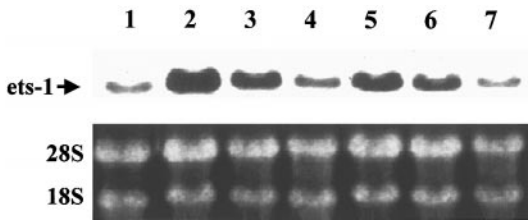

FIG. 4. Influence of transcription factor decoy on the expression of ETS-1. Double-stranded synthetic oligonucleotides of the HRE-like sequence (decoy) or a mutant oligonucleotide (mutant) was used to transfect T24 cells. Thereafter, the cells were exposed to hypoxia or treated with $CoCl_2$. The expression of ets-1 mRNA was determined by Northern blotting. Lane 1, normoxia; Lane 2, hypoxia; lane 3, hypoxia plus mutant; lane 4, hypoxia plus decoy; lane 5, $CoCl_2$; lane 6, $CoCl_2$ plus mutant; lane 7, $CoCl_2$ plus decoy.

lane 4). Cobalt, which mimics the hypoxic condition, induced the expression of ets-1 mRNA, and this induction was again inhibited by the decoy (Fig. 4, lanes 5 and 7). The transfection with a mutant double-stranded oligonucleotides had little effect (Fig. 4, lanes 3 and 6). From these results, we concluded that HIF-1 played an important role in the hypoxia-mediated induction of ETS-1.

An increasing amount of evidence suggests that a role for hypoxia in the pathophysiology of solid tumors. Indeed, tumor hypoxia is strongly associated with tumor propagation, malignant progression, tumor angiogenesis, and resistance to therapy, and it has thus become a central issue in tumor physiology and cancer treatment (16). HIF-1 is the principal transcription factor related to hypoxia, and it has been demonstrated that HIF-1 activity is increased in various tumors relative to that in normal tissues (17–19). Moreover, HIF-1 provides a molecular basis for VEGF-induced angiogenesis in tumors (20, 21), as well as adaptations of cancer cells to hypoxia that are critical for establishment of a primary tumor and its progression to the lethal phenotype (19).

ETS-1 is another transcription factor, and is reported to be related to tumor invasion and angiogenesis (2–11). Our present results indicate for the first time that ETS-1 is induced by the activity of HIF-1. The interaction between these two transcription factors may provide a clue for understanding the novel mechanisms understanding the modulation of the properties of cancer cells as well as angiogenesis.

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