Sp1-Dependent Regulation of the RTP801 Promoter and Its Application to Hypoxia-Inducible VEGF Plasmid for Ischemic Disease

Minhyung Lee,[1] Malavosklish Bikram,[2] Seungjoon Oh,[3] David A. Bull,[4] and Sung Wan Kim[2,5]

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Purpose. Gene therapy using vascular endothelial growth factor (VEGF) is a new potential treatment of ischemic disease. To be safe and effective, VEGF expression should be enhanced locally in ischemic tissue. In this study, we identified the cis-regulatory element for the hypoxia induction of the RTP801 promoter. In addition, pRTP801-VEGF was evaluated as a therapeutic plasmid *in vitro*.

Methods. The cis-regulatory element for hypoxia induction was identified by deletion and mutation analyses. Antisense oligonucleotide co-transfection assay was performed to evaluate the role of Sp1. pRTP801-VEGF was constructed by the insertion of the RTP801 promoter into the VEGF plasmid. The hypoxia-inducible expression of VEGF was evaluated by *in vitro* transfection assay.

Results. In luciferase assay, the region between –495 and –446 was responsible for the hypoxia-induced transcription. The mutation of the Sp1 site in this region reduced hypoxia-induced transcription. In addition, co-transfection with antisense Sp1 oligonucleotide suggests that hypoxia induction of the RTP801 promoter is mediated by Sp1. *In vitro* transfection showed that pRTP801-VEGF had higher VEGF expression than pEpo-SV-VEGF. In addition, VEGF expression by pRTP801-VEGF was induced under hypoxia.

Conclusions. With strong basal promoter activity and induction under hypoxia, pRTP801-VEGF may be useful for gene therapy for ischemic disease.

KEY WORDS: hypoxia; RTP801; Sp1; transcriptional regulation; vascular endothelial growth factor.

INTRODUCTION

Gene therapy with VEGF is a new potential treatment of ischemic diseases such as ischemic heart disease. The delivery of the VEGF gene to ischemic heart has been achieved by naked DNA injection, polymeric carriers, retrovirus adenovirus, or adeno-associated virus (1–3). Naked DNA injection is safe, as it does not have cytotoxicity or severe immune response. The previous reports have shown that naked plasmid delivery of the VEGF gene is useful for the treatment of ischemic myocardium (4–6). However, naked plasmid requires a large amount of administration due to the low efficiency of gene expression (3). To improve the plasmid delivery efficiency, polymeric gene carriers have been developed. These polymeric gene carriers include TerplexDNA and water-soluble lipopolymer (WSLP) (7,8). These polymeric gene carriers increased the transfection efficiency to myocardium up to 10-fold. In addition, the duration of the gene expression was prolonged compared to naked DNA. The prolonged duration of the gene expression by polymeric carriers may be due to the ability of the carriers to protect from nucleases. The virus-mediated gene transfer showed high gene transfer and expression activity. It is generally accepted that viral carrier is the most efficient way to transfer the therapeutic genes. However, virus-mediated gene transfer has potential immunogenecity or host chromosomal integration, suggesting possible mutagenesis (1).

Therapeutic angiogenesis is a new potential treatment in cardiovascular disease and hindlimb ischemia. Therapeutic angiogenic therapy is performed by the delivery of the angiogenic agent. Currently, VEGF is the most effective therapeutic gene for neo-vascularization (3). Therapeutic angiogenesis using VEGF gene therapy has been established after preclinical and clinical studies. Previously, it was reported that both VEGF and its receptors were up-regulated in ischemic tissues (9). Therefore, it was suggested that ischemia is necessary for VEGF to enhance its effects (10). However, Springer *et al.* proved that exogenously delivered VEGF could exert a physiological effect in normal, non-ischemic tissue (11). In addition, unregulated continuous expression of VEGF is associated with formation of endothelial cell–derived intramural vascular tumors (12). This suggested that VEGF expression must be regulated. Therefore, Epo (erythropoietin) enhancer was used to enhance VEGF gene expression locally in ischemic tissues. Our group showed that Epo enhancer-SV40 promoter induced gene expression under hypoxia in human embryonic kidney 293 cells *in vitro* and in rabbit ischemic myocardium *in vivo* (13). In addition, Su *et al.* proved hypoxia-responsive element mediated VEGF expression in ischemic myocardium by using an adeno-associated virus (14). In this trial, the VEGF gene was regulated by HRE and SV40 promoter. This regulation of the VEGF expression system may be useful for safer VEGF gene therapy minimizing unwanted side effects.

The RTP801 promoter is an effective regulatory system for VEGF gene therapy. The previous report showed that RTP801 transcription was rapidly and sharply increased both *in vitro* and *in vivo* (15). This inducible expression of RTP801 is mediated by transcriptional activation. In addition, it was suggested that the induction of the RTP801 promoter was mediated by hypoxia-inducible factor-1 (HIF-1) (15). HIF-1 is a transcription factor that mediates hypoxia induction of a number of genes (16,17). Binding of HIF-1 to the consensus domain of the genes results in the transcriptional induction of the gene promoters (18,19). HIF-1–mediated induction of gene transcription is a widespread oxygen-sensing mechanism in various types of cells (20). Sp1-mediated hypoxia induction of gene transcription was recently proposed. The expression of cyclooxygenase-2 gene was increased by Sp1 (21).

In the current study, we analyzed the RTP801 promoter and identified a cis-regulatory element that was responsible for the hypoxia induction of the promoter. A potential Sp1

[1] Clinical Research Center, College of Medicine, Inha University, Inchon 400-711, Korea.
[2] Center for Controlled Chemical Delivery, Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, Utah, 84112, USA.
[3] Department of Internal Medicine, College of Medicine, Kyung Hee University, Seoul 130-701, Korea.
[4] Department of Surgery, Division of Cardiothoracic Surgery, University of Utah Health Sciences Center, Salt Lake City, Utah 84112, USA.
[5] To whom correspondence should be addressed. (e-mail: rburns@pharm.utah.edu)

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element in the RTP801 promoter was involved in hypoxia induction. We showed that the RTP801 promoter was induced under hypoxia condition in various types of cells. The VEGF plasmid including the RTP801 promoter induced VEGF expression under hypoxia. The results suggest that the hypoxia-inducible VEGF gene therapy using the RTP801 promoter is effective and safe, because it will minimize the side effect of the nonspecific VEGF expression and induce VEGF expression specifically in ischemic tissue. Therefore, the hypoxia-inducible VEGF plasmid, pRTP801-VEGF, may be a useful angiogenic gene therapy for ischemic diseases such as ischemic heart disease and hindlimb ischemia.

MATERIALS AND METHODS

Plasmids

The RTP801 promoter was cloned by polymerase chain reaction (PCR) using genomic DNA from HepG2 cells. The genomic DNA was extracted from HepG2 cells by using Qiagen DNeasy Tissue system (Qiagen, Valencia, CA, USA). The sequences of the specific primers for the RTP801 promoter were as follows: forward primer 5′-GAAGATCTAGCTTTAGGATCCAAGACGC-3′; backward primer 5′-CCCAAGCTTGGTGAGGACAGACGCCAGG-3′. Bgl II and HinD III sites were introduced to forward and backward primers, respectively, for cloning convenience (the enzyme sites are underlined). The PCR-amplified RTP801 (725 base) fragment was digested with Bgl II and HinDIII and purified by gel electrophoresis and elution. The pGL3-promoter plasmid was purchased from Promega (Madison, WI, USA). The SV40 promoter was eliminated by digestion with Bgl II and HinD III, and the plasmid backbone was purified by gel electrophoresis and elution. The RTP801 promoter fragment was inserted to pGL3-promoter, resulting in construction of pRTP801-725. The RTP801 promoter was confirmed by DNA sequencing.

The deletion of the 5′-region of the promoter was made by PCR. The backward primer was the same as above. The sequences of the forward primers were as follows: for RTP801-645, 5′-GAAGATCTCTGGTCACGGGCTGTCCCCT-3′; for RTP801-545, 5′-GAAGATCTCTGCAGCCGCCGCGGATCCT-3′; for RTP801-495, 5′-GAAGATCTGGTTCGACTGCGAGCTTTCT-3′; for RTP801-445, 5′-GAAGATCTGTCACCGGGCAGGAGAGAAC-3′; for RTP801-395, 5′-GAAGATCTCAAGGCGGGCCACACTCCCG-3′. The PCR fragments were subcloned into pGL3-promoter in the same method as described above. The cloning of each fragment was confirmed by DNA sequencing.

The Sp1 mutant constructs were generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) using pRTP801-725 construct as a template. The sequences of the mutated Sp1 oligonucleotides used are as follows: Sp1 mutant upstrand, 5′-CTGGGGCTCAATGGATTATGGGCCCGGCCGCTGT-3′; Sp1 mutant downstrand, 5′-ACAGCGGCCGGGCCCATAATCCATTGAGCCCCAG-3′.

To construct the VEGF plasmid, the RTP801 promoter was isolated by digestion of pRTP801-725 with Bgl II and HinDIII. pSV-VEGF was constructed as previously described (13). The RTP801 was inserted at the Bgl II and HinDIII sites of pSV-VEGF after deletion of the SV40 promoter.

Cell Cultures and Transfection

The 293, A7R5, NIH3T3, HepG2, and HUVE cells were purchased from ATCC. The 293, A7R5, NIH3T3, and HepG2 cells were maintained in DMEM supplemented with 10% FBS in a 5% CO_2 incubator. HUVEC were maintained in F-12K medium supplemented with 10% FBS, 2 mM L-glutamine, 1.5 mg/ml sodium bicarbonate, 0.1 mg/ml heparin, and 0.04 mg/ml endothelial cell growth supplement (ECGS) in a 5% CO_2 incubator.

For the transfection assays, the cells were seeded at a density of 5.0×10^5 cells/well in 35-mm cell culture dish (Falcon Co., Becton Dickenson, Franklin Lakes, NJ, USA) 24 h before transfection. Polyethylenimine (PEI, 25,000 Da) was used as a gene carrier. Plasmid/PEI complexes (2 μg plasmid/dish) were prepared at a 5/1 N/P ratio and incubated for 30 min at room temperature. The cells were washed twice with serum-free medium, and then 2 ml of fresh serum-free medium was added. The plasmid/PEI complex was added to each dish. The cells were then incubated for 4 h at 37°C in a 5% CO_2 incubator. After 4 h, the transfection mixtures were removed, and 2 ml of fresh medium containing FBS was added. The cells were incubated at the desired concentration of oxygen for 20 h. The cells and the media were harvested for luciferase assay and enzyme-linked immunosorbent assay (ELISA).

For co-transfection with the antisense oligonucleotides, the plasmid/PEI complex and the oligonucleotide/PEI complex (2 μg oligonucleotides/dish and 2 μg plasmid/dish) were separately prepared at a 5/1 N/P ratio and mixed. The transfection assay was carried out as described above. The sequences of the oligonucleotides were as follows: antisense Sp1, 5′-ATATTAGGCATCACTCCAGG-3′; sense Sp1, 5′-CCTGGAGTGATGCCTAATAT-3′; antisense HIF-1, 5′-GCCGGCGCCCTCCAT-3′; sense HIF-1, 5′-ATGGAGGGCGCCGGC-3′.

Luciferase Assay

After incubation, the cells were washed with PBS twice, and 150 μl of reporter lysis buffer (Promega) was added to each dish. After 15 min of incubation at room temperature, the cells were harvested and transferred to microcentrifuge tubes. After 15 s of vortexing, the cells were centrifuged at 11,000 rpm for 3 min. The extracts were transferred to fresh tubes and stored at -70°C until use. The protein concentrations of the extracts were determined by using a BCA protein assay kit (Pierce, Iselin, NJ, USA). Luciferase activity was measured in terms of relative light units (RLU) using a 96-well plate Luminometer (Dynex Technologies Inc, Chantilly, VA, USA). The luciferase activity was monitored and integrated over a period of 60 s. The final values of luciferase were reported in terms of RLU/mg total protein.

Enzyme-Linked Immunosorbent Assay

ELISA was performed using ChemiKine human vascular endothelial growth factor sandwich ELISA kit (Chemicon, Temecular, CA, USA). One hundred microliters of sample was added into the designated wells. Twenty-five microliters of biotinylated rabbit anti-human VEGF polyclonal antibody was added to each well, and the plate was incubated at room temperature for 3 h. After the incubation, the plate was

washed 7 times with wash buffer. Fifty microliters of the streptavidin-alkaline phophatase was added into each well, and the plate was incubated at room temperature for 45 min. After the incubation, the plate was washed 7 times with wash buffer. The substrate was added to the wells, and the absorbance was measured at 490 nm.

Statistical Analysis

The comparison of VEGF concentration was made by Student's *t* test. p value under 0.05 was thought to be statistically significant.

RESULTS

Deletion Analysis of the Responsiveness of the RTP801 Promoter to Hypoxia

To identify the region necessary for hypoxia induction in the RTP801 promoter, various fragments of the 5′-flanking region up to 725 bp of the RTP801 promoter upstream from the ATG translation initiation codon were cloned into the luciferase reporter gene plasmid (Fig. 1A). These constructs were transfected into 293 cells using polyethylenimine (PEI) as a gene carrier. The transfected cells were incubated under hypoxia (1% O_2) or normoxia (20% O_2) for 20 h. After incubation, the cell extracts were prepared from the transfected cells, and the luciferase activity was measured. As a result, the activities of pRTP801-725, pRTP801-645, pRTP801-545, and pRTP801-495 increased under hypoxia. However, the activity of pRTP801-445 did not increase (Fig. 1B). These results indicate that the cis-regulatory element that responds to hypoxia exists between –495 and –446. The luciferase activity of pRTP801-545 was lower than that of pRTP801-725 and pRTP801-495 (Fig. 1B). This suggests that there may be a negative regulatory element between –545 and –496. This element reduced the activity under both hypoxia and normoxia. The negative regulatory element was not identified in this study, because it is unlikely to regulate the promoter activity for the hypoxia induction.

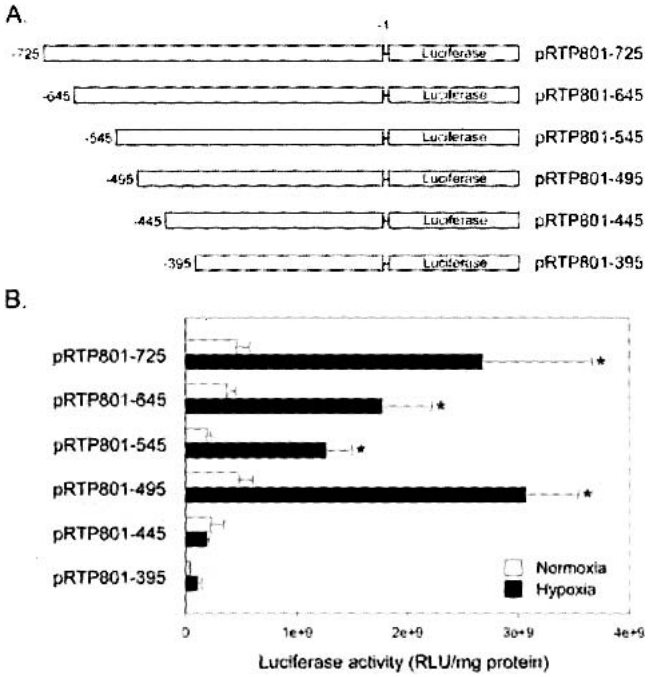

Fig. 1. (A) The structures of the pRTP801 luciferase reporter vectors. The diagram shows the structures of the pRTP801 luciferase reporter vectors containing various lengths of 5′-flanking regions of the human RTP801 promoter. (B) Hypoxia responsiveness of the 5′-flanking region of the RTP801 promoter. The reporter constructs were transiently transfected into the 293 cells. The cells were incubated for 24 h under normoxic or hypoxic conditions, and the luciferase activity was determined. The data is expressed as mean values (±SD) of four experiments. *p < 0.01 as compared to normoxia.

Role of Sp1 in the Induction of the RTP801 Promoter

Sequence analysis showed that there was a potential Sp1 consensus binding site in the region between –495 and –446. To evaluate the effect of mutation of this Sp1 binding site, site-directed mutagenesis was performed. The sequence of GGCG (–459 ~ –462) was replaced with the sequence of TTAT, resulting in construction of pRTP801-SP1(–) (Fig. 2A). pRTP801-725 (wild-type) and pRTP801-SP1(–) were transfected into 293 cells, and the transfected cells were incubated under hypoxia or normoxia condition. After the incubation, the luciferase activity was measured. In the cells transfected with pRTP801-SP1(–), the hypoxia induction of the RTP801 promoter decreased, compared to the cells transfected with pRTP801-725 (Fig. 2B). Therefore, this result indicates that the Sp1 element in the RTP801 promoter plays an important role in the hypoxia induction of the RTP801 promoter.

Another approach to show that Sp1 is important for hypoxia induction of the RTP801 promoter was to use antisense oligonucleotides directed against Sp1 (Fig. 3). The antisense Sp1 oligonucleotides were co-transfected with pRTP801-725 into 293 cells. As a control, the sense Sp1 oligonucleotides

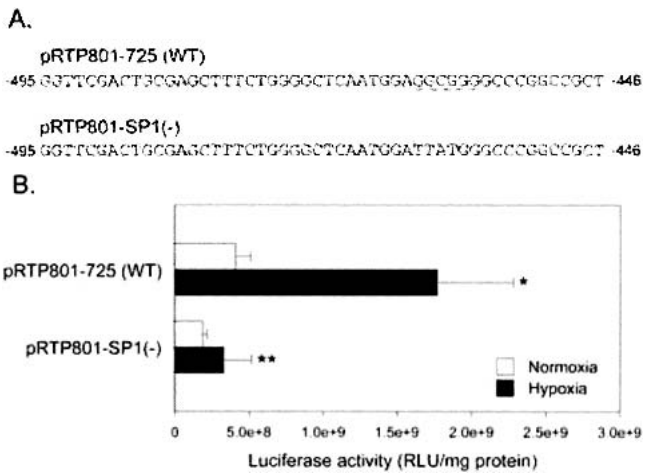

Fig. 2. (A) Sequence of the RTP801 promoter between –495 and –446. The Sp1 consensus sequence of the RTP801 promoter in the pRTP801-725 is underlined. The sequence of the pRTP801-Sp1(–) is also shown. The mutation position is indicated by rectangle. (B) Effect of mutation in Sp1 element in the RTP801 promoter on the promoter activity. pRTP801-725 and pRTP801-Sp1(–) were transiently transfected into the 293 cells. The cells were incubated for 20 h under normoxic or hypoxic conditions, and the luciferase activity was determined. The data is expressed as mean values (±SD) of four experiments. *p < 0.01 as compared to normoxia. **No statistical significance as compared to normoxia.

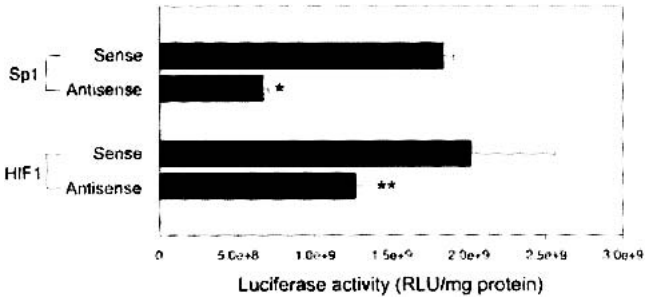

Fig. 3. Role of Sp1 in the hypoxia-inducibility of the RTP801 promoter. pRTP801-725 was co-transfected into the 293 cells with Sp1 sense, Sp1 antisense, HIF-1 sense, or HIF-1 antisense oligonucleotides. The cells were exposed to hypoxia for 20 h and assessed for luciferase activity. The data is expressed as mean values (±SD) of four experiments. *$p < 0.01$ as compared to Sp1 sense. **$p < 0.05$ as compared to HIF-1 sense.

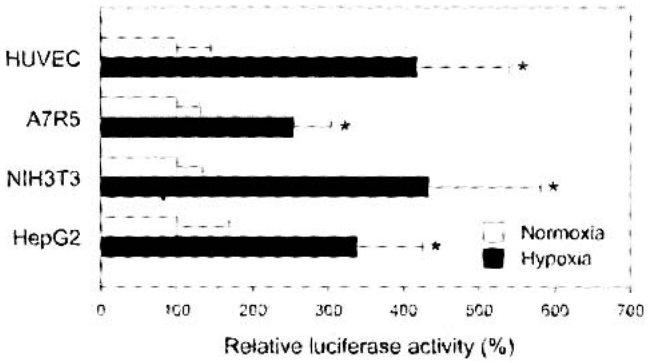

Fig. 4. Hypoxia-inducibility of the RTP801 promoter in various cell lines. pRTP801-725 was transfected into HUVE, A7R5, NIH3T3, or HepG2 cells. The cells were exposed to hypoxia or normoxia for 20 h and assessed for luciferase activity. The data expressed as mean values (±SD) of three experiments. *$p < 0.05$ as compared to normoxia.

were co-transfected with pRTP801-725. The antisense HIF1 or sense HIF1 oligonucleotides were also co-tranfected with pRTP801-725 to evaluate the effect of HIF-1. The transfected cells were incubated under hypoxia for 20 h. The cell extracts were prepared from the cells, and the luciferase activity was measured. As a result, in the cells transfected with the antisense Sp1 oligonucleotides, the activity of the RTP801 promoter was reduced, compared to the sense Sp1 oligonucleotides transfected cells (Fig. 3). This result suggests that Sp1 mediates hypoxia induction of the RTP801 promoter. On the other hand, in the cells transfected with the antisense HIF-1 oligonucleotides, the activity of the RTP801 promoter decreased, suggesting the role of HIF-1. This result is consistent with the previous report, which suggested that HIF-1 was involved in the hypoxia induction of the RTP801 promoter (15).

The Transcriptional Induction of the RTP801 Promoter in Various Cell Lines

To apply the RTP801 promoter to gene therapy in various organs, we had to confirm that the RTP801 promoter did not have cell-type specificity in the gene expression. The first evidence was provided in the previous study (15), which showed that the RTP801 gene was expressed in various organs at basal level. Therefore, we hypothesized that the RTP801 promoter can be induced in the various types of cells. To test this hypothesis, we transfected pRTP801-725 into various types of cells, including HUVEC (human umbilical vascular endothelial cell), A7R5 (rat smooth muscle cell), NIH3T3 (mouse fibroblast cell), and HepG2 (human hepatocyte). The luciferase assay showed that the RTP801 promoter induced the gene expression under hypoxia by about 2- to 4-fold in various types of cells (Fig. 4).

The VEGF Expression Mediated the RTP801 Promoter

To apply the RTP801 promoter to VEGF gene therapy, we constructed pRTP801-VEGF by the insertion of the RTP801 promoter upstream of the VEGF cDNA (Fig. 5). pEpo-SV-VEGF, which had been constructed previously, was used as a positive control plasmid (13). The previous report showed that pEpo-SV-VEGF induced the VEGF expression effectively in hypoxic 293 cells and the rabbit ischemic myocardium, suggesting the possibility of the application of this system to gene therapy. pRTP801-VEGF and pEpo-SV-VEGF were transfected into 293 cells using PEI as a gene carrier. pSV-Luc was transfected as a negative control. The transfected cells were incubated under hypoxia for 20 h. The cell culture media was collected, and the expression level of the VEGF gene was measured by ELISA. As a result, the VEGF expression level was induced in both the pRTP801-VEGF and pEpo-SV-VEGF transfected cells (Fig. 6). However, the basal or induction level of VEGF expression by the RTP801 promoter was higher than that by the Epo enhancer-SV40 promoter.

DISCUSSION

In the current study, we focused on two points: the transcriptional induction mechanism of the RTP801 promoter and the evaluation of the RTP801 promoter–driven VEGF plasmid as a therapeutic plasmid. In the first part, we have shown that the hypoxia responsiveness of the RTP801 promoter was mediated by Sp1 element in 293 cells. Second, the hypoxia responsiveness of the RTP801 promoter was widespread in various cell lines, which is favorable for gene therapy application of the promoter. Furthermore, the basal level or hypoxia induction of VEGF by the RTP801 promoter was stronger than that by the Epo enhancer-SV40 promoter system.

The RTP801 protein was suggested to be involved in the

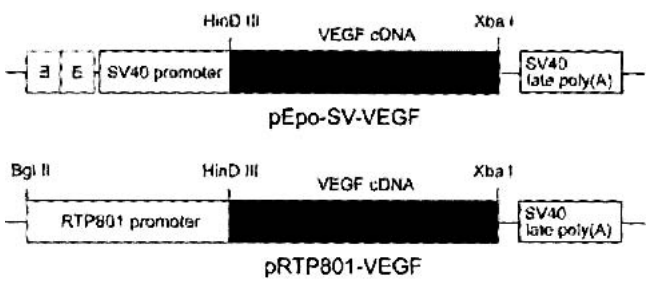

Fig. 5. The structures of pEpo-SV-VEGF and pRTP801-VEGF. In the pEpo-SV-VEGF, two copies of the Epo enhancers were inserted upstream of the SV40 promoter. In the pRTP801-VEGF, the RTP801 promoter was inserted upstream of the VEGF cDNA. E represents the Epo enhancer.

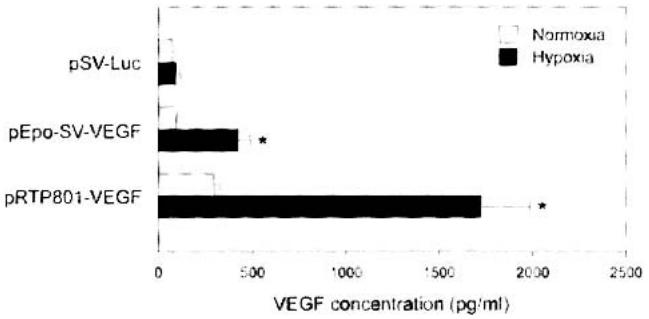

Fig. 6. The induction of VEGF expression. pEpo-SV-VEGF and pRTP801-VEGF were transfected into the 293 cells. pSV-Luc was transfected as a negative control. The cells were exposed to normoxia or hypoxia for 20 h. After the incubation, the cell culture media was collected, and the VEGF concentration was determined by ELISA. The data is expressed as mean values (±SD) of three experiments. *$p < 0.01$ as compared to normoxia.

hypoxia-responsive apoptosis mechanism (15). However, the detailed mechanism and function of RTP801 have not been fully identified. It was suggested that the hypoxia induction of the RTP801 promoter was mediated by HIF-1 transactivation mechanism at least in mouse ES cell line. In the current study, we suggest that the hypoxia induction of the RTP801 promoter is mediated by Sp1, at least in 293 cells. Therefore, it is likely that the RTP801 promoter is regulated by Sp1 and HIF-1. The mechanism, which is mediated by HIF-1, has been intensively studied by a number of researches. Mainly, the HIF-1 transcription factor is stabilized under hypoxia condition. This stabilized mechanism is mediated by ODD (oxygen dependent degradation) domain, which degrades the HIF-1 transcription factor under normoxia by ubiquitin-mediated process (22). However, the Sp1-mediated induction mechanism of the promoter activation has not been fully identified. Previously, the possibility of the hypoxia induction of Sp1 was suggested by Xu *et al.* (21). In this study, they suggested that cycloocygenease-2 promoter was induced by Sp1 in hypoxic vascular endothelium (21). In addition, it was suggested that the binding level of Sp1 increased in response to hypoxic condition.

In the RTP801 promoter, the relation between Sp1 and HIF-1 is not yet identified. However, it is possible that Sp1 and HIF-1 cooperate to induce the RTP801 promoter under hypoxia. One example of cooperation between Sp1 and HIF-1 was recently suggested in the endoglin transcription under hypoxia (23). It was proposed that the endoglin transcription be induced under hypoxia through Sp1 interaction with HIF-1 and Smad transcription factors (23). In a multiprotein complex (Sp1-Smad3-HIF-1) on the endoglin promoter, Smad3 appears to function not only as a co-activator factor, but also as an adaptor between HIF-1 and Sp1 (23).

To identify the applicability of the RTP801 promoter to the hypoxia-inducible VEGF gene for gene therapy, we tested two criteria. First, the promoter should be active and inducible in various cell lines, which can broaden the gene therapy application possibilities. We showed that the RTP801 promoter was active and inducible in 293 cells, NIH3T3 cells, HepG2 cells, A7R5 cells, and HUVEC. Second, the promoter activity should be strong enough to express a large amount of therapeutic gene. We used the Epo enhancer-SV40 promoter as a control system. Previously, this Epo enhancer-SV40 promoter system was evaluated in hypoxic 293 cells and the rabbit ischemic myocardium, suggesting the possibility of the application of this system (13). In addition, the HRE-SV40 promoter system was proven in mouse ischemic myocardium previously *in vivo*. In the current study, the RTP801 promoter had a stronger promoter activity than Epo enhancer-SV40 promoter system. The hypoxia induction fold of the RTP801 promoter was not higher than that of Epo enhancer-SV40 promoter system, which was about 5-fold in both promoter systems. However, the RTP801 promoter had the higher basal level promoter activity compared to the Epo enhancer-SV40 promoter system, resulting in the higher expression of VEGF under both hypoxia and normoxia. It is well-known that nonviral gene carriers have low transfection level compared to viral carriers, which is often not sufficient for the clinical purposes. Therefore, it has been desirable to develop efficient nonviral gene carriers. In addition, the more efficienct gene expression system empolying promoters, introns, 5′- or 3′-untranslated regions would be beneficial to nonviral gene therapy systems. Therefore, the RTP801 promoter may be useful to nonviral gene therapy, as it was proven that this promoter was stronger than the Epo enhancer-SV40 promoter in the current study. Furthermore, hypoxia induction of VEGF by the RTP801 promoter will be beneficial to both nonviral and viral gene delivery.

In summary, the RTP801 promoter was induced under hypoxia condition by an Sp1-mediated mechanism. In addition, the promoter activity of the RTP801 promoter was higher than Epo enhancer-SV40 promoter system, suggesting a possible application to ischemic disease gene therapy. A model system, which includes the RTP801 promoter and the VEGF cDNA, could successfully induce the VEGF expression under hypoxia. Therefore, this RTP801 promoter may be useful for the development of a hypoxia-inducible gene therapy system for ischemic diseases.

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