Gene Therapy Targeting for Hepatocellular Carcinoma: Selective and Enhanced Suicide Gene Expression Regulated by a Hypoxia-inducible Enhancer Linked to a Human α-Fetoprotein Promoter

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ABSTRACT

We previously reported that the retroviral vector expressing the herpes simplex virus-thymidine kinase gene under the control of 0.3-kb human α-fetoprotein (AFP) gene promoter (AF0.3) provided the cytotoxicity to ganciclovir (GCV) in high-AFP-producing human hepatoma cells but not in low-AFP-producing cells. Therefore, specific enhancement of AFP promoter activity is likely to be required to induce enough cytotoxicity in low-AFP-producing hepatoma cells. In this study, we constructed a hybrid promoter, [HRE]AF, in which a 0.4-kb fragment of human vascular endothelial growth factor 5'-flanking sequences containing hypoxia-responsive element (HRE) was fused to AF0.3 promoter. By means of the reporter gene transfection assay, hypoxia-inducible transcriptions that were mediated by [HRE]AF promoter were detected in low- and non-AFP-producing human hepatoma cells, but not in nonhepatoma cells. When the herpes simplex virus-thymidine kinase gene controlled by [HRE]AF promoter was transduced into hepatoma and nonhepatoma cells by a retroviral vector, the exposure to 1% O2 induced GCV cytotoxicity specifically in the hepatoma cells. Moreover, in nude mice bearing solid tumor xenografts, only the tumors consisting of the virus-infected hepatoma cells gradually disappeared by GCV administration. These results indicate that the hypoxia-inducible enhancer of the human vascular endothelial growth factor gene, which is directly linked to human AFP promoter, involves selective and enhanced tumoricidal activity in gene therapy for hepatocellular carcinoma.

INTRODUCTION

HCC is one of the most common malignancies worldwide, especially in several areas of Asia and Africa. Because the development of HCC is strongly associated with chronic liver disease, particularly cirrhosis that occurs as a result of hepatitis B virus or HCV, the close follow-up of patients with chronic hepatitis B virus or HCV infection using imaging techniques and serum AFP assays has led to the detection of HCC at an early stage. However, even among patients in whom HCC is detected early, there are very few candidates for surgery because they generally lack a hepatic reserve as a result of the coexisting advanced cirrhosis. Moreover, clinical observations have shown that tumor recurrence rates are very high in patients with HCC who receive medical or surgical treatments. Thus, new treatment modalities must be pursued.

One new approach involves gene therapy using a retroviral vector that carries a kill or suicide gene such as the HSV-tk gene (4–6). Retroviruses can stably integrate their genes into proliferating cells, but they cannot integrate into quiescent cells. The killing effect of the HSV-tk gene product on the virus-infected cells is seen only in proliferating cells in which HSV-tk can efficiently phosphorylate nucleoside analogues and in which the phosphorylated products act as a chain terminator of DNA synthesis, thus leading to cell death (7–9). If the HSV-tk gene is driven by the tumor-specific promoter, it can cause selective ablation of tumor cells.

Many cancers often reexpress fetal or embryonic genes, and AFP gene expression is reactivated in HCC cells (10). There has been much progress in the characterization of cis- and trans-acting elements regulating human AFP gene expression (11–13). The hepatocyte-specific enhancers exist in a far upstream-regulatory region (−3.7- and −3.5-kb) of the AFP gene, and the position-dependent silencers are located between the enhancer regions and the hepatocyte-specific promoter region. To improve the selectivity of the antitumor effect in gene therapy for HCC, most investigators have used human AFP 5'-flanking sequences including enhancers or both enhancers and a silencer (4, 14–17). We previously reported on hepatoma-specific gene therapy using an AFP promoter alone to target AFP-producing hepatoma cells (5, 18, 19). The retroviral vector expressing the HSV-tk gene under the control of 0.3-kb human AFP gene promoter (AF0.3) provided cytotoxicity to GCV in high-AFP-producing human hepatoma cells (5). In contrast, the retroviral infection had little effect on the low-AFP-producing cells, because the 0.3-kb AFP promoter activity alone was not enough to express the HSV-tk gene in these cells. Because the level of AFP expression mostly depends on the activity of the enhancer, our AFP promoter/HSV-tk approach could decrease the cytotoxicity of GCV to stem cells, even if they express AFP, resulting in protection of the hepatic reserve. Recently, it has become possible to clinically detect HCCs at an early stage, and AFP expression in these tumors has been found to be relatively low (2). Furthermore, Northern blot analysis revealed only slight AFP expression in a noncancerous cirrhotic liver (20). Therefore, hepatoma-specific enhancement of AFP promoter activity is likely to be required to induce enough cytotoxicity in low-AFP-producing hepatoma cells.

Tumor development requires oxygen and nutrients, which are supplied through neovascularization. Angiogenic potential is, therefore, a prerequisite for tumor growth. Several growth factors (21–23), inflammatory cytokines (24, 25), and angiogenin (26) promote tumor angiogenesis, with the growth factors thought to be the most important of these. VEGF is vital for the neovascularization associated with tumor progression (27, 28). The expression of VEGF is induced by hypoxia through the HRE that exists in the VEGF 5'-flanking region (29). Recently, higher levels of VEGF expression have been found in HCC, which is generally considered to be a hypervascular tumor, than in corresponding nontumorous tissues (30–32).

In this study, we constructed a hybrid promoter ([HRE]AF) consisting of the hypoxia-inducible enhancer of the human VEGF gene. This hybrid promoter contained HRE directly linked to AF0.3 promoter. We evaluated the hepatoma-specific enhancement of GCV-
mediated cytotoxicity that was induced by the HSV-\(k\) gene under the control of [HRE]AF promoter in vitro and, more importantly, in vivo.

MATERIALS AND METHODS

Cell Culture. The amphotropic retrovirus packaging line PA317 was maintained in DMEM supplemented with 5% calf serum. The human hepatoma (PLC/PRF/5 and HLF) and cervical cancer (HeLa) cell lines were cultured in RPMI 1640 with 5% FBS, and the human fibrosarcoma (HT1080) was cultured in RPMI 1640 with 10% FBS.

All cell lines were incubated in a humidified 5% CO\(_2\) and 95% air incubator at 37°C (normoxia). Hypoxia (1% O\(_2\)) was induced by placing cells in a 1% O\(_2\)-5% CO\(_2\) and 94% N\(_2\) incubator for 24 h at 37°C.

Luciferase Fusion Plasmids. The 1.8-kb fragment containing a 5′-flanking region and exon 1 of the human VEGF gene was cloned by PCR using 5′-ATTGTCGTCCATTCCATCTAGCA-3′ and 5′-GGGATGCCAAGCATAA-3′ as primers under the following conditions: denaturation at 94°C for 30 s; annealing at 55°C for 1 min; and extension at 72°C for 1 min (30 cycles). The 1.8-kb PCR products that corresponded to −1.6 kb to +0.2 kb of the human VEGF gene were cloned into pCR2.1 (Invitrogen, Carlsbad, CA) to make pCR-VEGFpro and then sequenced. The nucleotide sequence of the PCR product was identical with that of the human VEGF gene previously deposited with GenBank/EMBL Data Bank under accession no. M63971 (33).

To make luc fusion plasmids, pVE1.3-, pVE1.2- and pVE0.4-luc, the −1.3-, −1.2-, and −0.4-kb of the VEGF 5′-flanking region were released by BamHI-, SacI-, and BglII-HindIII double digestions, respectively, and inserted into the BglII- or SacI-HindIII sites of pGL3-Basic, a firefly luc reporter vector (Promega, Madison, WI; Fig. 1A). To construct pVE0.8-luc, the −0.8-kb of the 5′-flanking region was released by PstI-HindIII digestion, and the PstI site of this fragment was blunt-ended. The resultant fragment was inserted into the Smal-HindIII site of the pGL3-Basic vector.

Human AFP gene 0.3-kb promoter was released from pBS-AF0.3 (5) by BamHI-HindIII digestion, and inserted into the BglII-HindIII site of pGL3-Basic vector, resulting in pAF0.3-luc (Fig. 2A). The 0.4-kb SacI-PstI fragment between −1.2 and −0.8 kb relative to the cap site of human VEGF gene containing HRE was inserted into the Smal site at the 5′-end of the AFP promoter of pAF0.3-luc and the SV40 promoter of the pGL-promoter vector (Promega), called pSV-luc in this study, resulting, respectively, in p[HRE]AF-luc and p[HRE]SV-luc (Fig. 2A).

Cell Transfection and Dual-Luciferase Reporter Assays. Cotransfection was performed using 1 μg of the luc fusion plasmids and 0.1 μg of pRL-TK, a Renilla luc control vector (Promega), per well in 24-well multiplates by lipofection methods (34). After transfection, the cells were incubated with the fresh medium under either the normoxic or hypoxic (1% O\(_2\)) condition. After 24 h of incubation, the cells were harvested and a Dual-Luciferase Reporter Assay was performed according to the instructions provided with the assay.

Retroviral Vectors. The retroviral vector, pLNAF0.3TK, which carries the HSV-\(k\) gene under the control of human AFP 0.3-kb promoter, was produced as described previously (5). To create pLN[HRE]AFTK, the human AFP promoter of pLNAF0.3TK was replaced with [HRE]AF promoter, which was released from p[HRE]AF-luc (Fig. 3).

Production of Amphotropic Retroviral. Amphotropic retroviruses were produced as described previously (35). Briefly, the retroviral vectors pLNAF0.3TK and pLN[HRE]AFTK were transfected into PA317 amphotropic packaging cells using calcium phosphate precipitation. The cells were selected in the medium with 800 μg/ml G418 (Geneticin; Wako, Osaka, Japan), and G418-resistant colonies were cloned using cloning rings.
RESULTS

Hypoxia-inducible Transcription Mediated by Human VEGF 5′-flanking Sequences in Hepatoma and Nonhepatoma Cells. To determine whether the 5′-flanking region of the VEGF gene mediates the transcriptional responses to cellular hypoxia, we constructed reporter plasmids in which VEGF 5′-flanking sequences were fused to luc coding sequences (Fig. 1A). This series of 5′ deletion mutants, pVE1.3-, pVE1.2-, pVE0.8-, and pVE0.4-luc, was transfected into human hepatoma (PLC/PRF/5 and HLF) and nonhepatoma cells (HT1080 and HeLa). After the cells were incubated in normoxia or hypoxia (1% O₂) for 24 h, a Dual-Luciferase Reporter Assay was performed.

The results are shown in Fig. 1B. pVE1.3- and pVE1.2-luc reporter plasmids mediated 2.5- to 16-fold greater levels of luc expression in both hepatoma and nonhepatoma cells that were exposed to hypoxia (1% O₂) than did cells that were exposed to normoxia. In contrast, pVE0.8- and pVE0.4-luc induced 60 to 80% less transcription than pVE1.3- and pVE1.2-luc in hypoxic cells, although, in comparison to cells exposed to normoxia, the luc expression mediated by pVE0.8- and pVE0.4-luc was weakly increased by hypoxia. These results demonstrate that VEGF 5′-flanking sequences mediate the transcriptional response to hypoxia in both hepatoma and nonhepatoma cells and that the region between 1.2 and 0.8 kb 5′ to the VEGF transcription initiation site is specifically involved in mediating hypoxia-inducible transcription, acting as the hypoxia-inducible enhancer. These results also agree with a previous report that described HRE as existing -1.0 kb upstream of the human VEGF gene (29).

Hepatoma-specific and Enhanced Transcriptional Activity of [HRE]AF Promoter. To obtain hypoxia-inducible and hepatoma-specific transcription, we prepared a hybrid promoter, [HRE]AF, in which the 0.4-kb fragment between 1.2 and 0.8 kb 5′ to the VEGF transcription initiation site was directly linked to the human AFP 3′-k promoter, AF0.3 (Fig. 2A). By transient transfection assay, transcription induced by AF0.3 or [HRE]AF was analyzed in human hepatoma or nonhepatoma cells under either normoxic or hypoxic conditions.

As shown in Fig. 2B, in PLC/PRF/5 cells (low-AFP-producing hepatoma cells) exposed to 1% O₂, the luc expression mediated by [HRE]AF promoter 22-fold greater than that mediated by AF0.3 promoter, although the addition of the VEGF hypoxia-inducible enhancer to AF0.3 also resulted in a 4-fold increase in luc expression in the normoxic cells. In HLF cells (non-AFP-producing hepatoma cells) without 1% O₂ exposure, both AF0.3 and [HRE]AF promoter slightly induced luc expression. However, in hypoxic HLF cells, [HRE]AF promoter mediated an increase in luc expression that was 100-fold greater than that mediated by AF0.3 promoter. The levels of luc expression induced by the [HRE]AF promoter were similar to those induced by the [HRE]SV promoter in these hypoxic hepatoma cells. In nonhepatoma cells, HT1080 and HeLa, luc expression driven by SV and [HRE]SV promoters was detected, and the expression of [HRE]SV was enhanced by 1% O₂ exposure, whereas transcription mediated by AF0.3 and [HRE]AF was not detected in the normoxic or hypoxic cells. These results indicate that hypoxia-inducible transcription through the VEGF enhancer directed by AF0.3 promoter is circumscribed within hepatoma cells, even if they do not express AFP, although the VEGF enhancer is functional in both hepatoma and nonhepatoma cells.

Specific and Enhanced Cytotoxicity of GCV in LN[HRE]AFTK Virus-infected Hepatoma Cells by Hypoxia. We constructed hybrid genes consisting of HSV-tk genes under the control of either AF0.3 or [HRE]AF promoter and inserted them into retroviral vectors, thus producing LNAF0.3TK and LN[HRE]AFTK, respectively (Fig. 3). The low- and non-AFP-producing hepatoma cells (PLC/PRF/5 and HLF, respectively) and nonhepatoma cells (HT1080 and HeLa) were infected with these recombinant retroviruses. Because the transduction efficiency might contribute to the results, G418-resistant pooled populations were used for in vitro and in vivo studies of GCV-mediated cytotoxicity to precisely evaluate the activity and specificity of the promoters.

LNAF0.3TK and LN[HRE]AFTK virus-infected cells were exposed to both 1% O₂ for 24 h every 2 days and to varying concen-
trations of GCV for 6 days. Their survival curves are shown in Fig. 4. LNAF0.3TK infection induced weak GCV cytotoxicity only in low-AFP-producing hepatoma cells (PLC/PRF/5), and induced no cytotoxicity in non-AFP-producing hepatoma cells (HLF) and nonhepatoma cells (HT1080 and HeLa). In contrast, LN[HRE]AFTK infection sensitized both low- and non-AFP-producing hepatoma cells to GCV but did not sensitize nonhepatoma cells.

These results demonstrate that the HSV-tk/GCV system under the control of [HRE]AF promoter is able to induce enhanced hepatoma-specific cytotoxicity in hypoxic cells.

**Hepatoma-specific Induction of HSV-tk/GCV System in Tumor-bearing Athymic Mice.** Tumor cells are exposed to hypoxia in solid tumors. Therefore, we analyzed LNAF0.3TK or LN[HRE]AFTK retrovirus-mediated cytotoxicity to GCV in athymic mice bearing solid tumor xenografts. Because the PLC/PRF/5 and HT1080 cells were transplatable in athymic mice, the LNAF0.3TK or LN[HRE]AFTK virus-infected tumor cells were inoculated s.c. in the mice. At 1 week after s.c. inoculation, the mice were given GCV for 14 days. The LNAF0.3TK virus-infected PLC/PRF/5 cells grew rapidly, regardless of GCV administration, reaching tumor volumes that were 5–6-fold greater during the observation period than during pretreatment (Fig. 5). The tumors consisting of LN[HRE]AFTK virus-infected PLC/PRF/5 cells gradually disappeared as a result of the 14-day GCV treatment, but without GCV administration they grew as rapidly as the LNAF0.3TK virus-infected PLC/PRF/5 cells (Fig. 5).

On the other hand, the growth of s.c. tumors consisting of LNAF0.3TK or LN[HRE]AFTK virus-infected HT1080 cells was not inhibited by the 14-day GCV administration.

These results indicate that the [HRE]AF/HSV-tk approach induces specific enhancement of the cytotoxicity to GCV in solid tumors made up of low-AFP-producing hepatoma cells.

**DISCUSSION**

In the present study, we transduced the HSV-tk gene under the control of the human AFP promoter (AF0.3) or a hybrid promoter ([HRE]AF) into low- and non-AFP-producing hepatoma cells and into nonhepatoma cells using the LNAF0.3TK and LN[HRE]AFTK retroviruses, respectively. The cytotoxicity to GCV that was induced by LNAF0.3TK infection was, in vitro, detected weakly in low-AFP-producing hepatoma cells, and not at all in non-AFP-producing hepatoma and nonhepatoma cells. In addition, the growth of tumor xenografts of LNAF0.3TK virus-infected low-AFP-producing hepatoma cells in nude mice was not affected by systemic GCV treatment for 14 days. On the other hand, the LN[HRE]AFTK infection induced GCV cytotoxicity in low- and non-AFP-producing hepatoma cells exposed to 1% O₂ and, when mice bearing solid tumors consisting of LN[HRE]AFTK-infected low-AFP-producing hepatoma cells were treated by GCV administration, a complete regression of the tumors was observed without any signs of overt toxicity. Importantly, the LN[HRE]AFTK infection did not sensitize nonhepatoma cells to GCV either in vitro or in vivo, although the VEGF hypoxia-inducible enhancer was functional in these cells that had been exposed to 1% O₂. The 0.3-kb AFP promoter contains a glucocorticoid response element and two binding sites for HNF-1, a hepatocyte-specific transcriptional factor, and a TATA box (5, 13, 38) but does not have a typical CCAAT sequence (39). Recent studies reported that one of the HNF-1 binding sites (−132 bp to −116 bp) was also recognized by NF-1 and CCAAT/enhancer binding proteins that function actively during postnatal liver growth (40, 41). Although NF-1 competes with HNF-1 in the HNF-1 binding site in non-AFP-producing hepatoma cells as well as in adult hepatocytes, the AFP promoter shows a trace of activity in non-AFP-producing hepatoma cells (42). Therefore, the VEGF hypoxia-inducible enhancer can selectively activate the AF0.3 promoter under the hypoxic condition in HLF non-AFP-producing hepatoma cells but not in HT1080 and HeLa nonhepatoma cells.

Because HCC frequently occurs in patients with liver cirrhosis and the prognosis of patients is influenced by the hepatic reserve, the selectivity of antitumor effects is vital for gene therapy for HCC. To improve selectivity, gene therapy based on targeted gene transfer or the specific expression of transfected genes to hepatoma cells is currently being investigated. However, most investigators have used human AFP 5' flanking region including the enhancers and silencer (4, 14–17). We previously reported on hepatoma-specific gene therapy using the 0.3-kb AFP promoter alone to target AFP-producing hepatoma cells (5, 18, 19). Also, we demonstrated that a human AFP
enhancer directly linked to its promoter and a variant type of AFP promoter with G-to-A substitution at nucleotide −119 was able to sensitize low-AFP-producing hepatoma cells to GCV (43, 44). However, the addition or modification of trans- and cis-acting elements from AFP 5′-flanking sequences, to achieve sufficient cytotoxicity in low-AFP-producing hepatoma cells, must also induce a killing effect on hepatic stem cells that also express AFP and appear in the injured liver, thus resulting in damage of the hepatic reserve and a poor prognosis for patients with HCC.

Aggressive tumors often have an insufficient blood supply, partly because tumor cells grow faster than the endothelial cells that make up the blood vessels and partly because the newly formed vascular supply is disorganized. This situation produces areas with reduced oxygen tension and nutrient deprivation. Hypoxia is a powerful modulator of gene expression. An important mediator of these responses is the interaction of a transcriptional complex termed HIF-1 with its cognate DNA recognition site, HRE. Molecular analysis of HIF-1 revealed that the DNA-binding complex consists of a heterodimer of two basic helix-loop-helix proteins, HIF-1α and HIF-1β (45). HIF-1α was a newly described protein, but HIF-1β had already been recognized as the dimerization partner of the aryl hydrocarbon receptor in the xenobiotic response, where it was termed the aryl hydrocarbon receptor nuclear translocator (46). Recently, several investigators have shown the potential of exploiting tumor-specific conditions for the targeted expression of therapeutics in cancer therapy (47–51). Dachs et al. (47) fused three copies of HRE from the mouse phosphoglycerate kinase-1 gene to the 9–27 promoter to control the expression of the bacterial cytoxin deaminase or the marker, CD2, gene. When HT1080 cells were transfected with the cytosine deaminase or CD2 gene under the control of the phosphoglycerate kinase-1 HRE, these cells were found to be more sensitive to the produrg 5-fluorocytosine than were the parental cells after exposure to hypoxia, and the CD2 expression was up-regulated in a solid tumor xenograft in nude mice. In another study, Gazit et al. (48) reported on starvation-inducible suicide gene therapy under the control of glucose-regulated protein 78 promoter, using a murine fibrosarcoma model. The strategies described in these reports are designed to use tumor-specific conditions, such as hypoxia or starvation, that exist in almost all solid tumors regardless of their origin or location, to control the expression of heterologous genes. In the approach described in the present study, when the VEGF hypoxia-inducible enhancer was directly linked to the AFP3 promoter, specific enhancement of transfected gene expression was induced in hypoxic hepatoma cells in vitro and in solid tumor xenografts consisting of low-AFP-producing hepatoma cells. However, hypoxia-inducible enhancement of AFP promoter activity mediated by the VEGF enhancer was not detected in nonhepatoma cells in vitro or in vivo. These results demonstrate that hypoxia-inducible enhancement of transgene expression through VEGF enhancer could be directed to be hepatoma specific by AFP promotor.

We used the 0.4-kb fragment containing HRE between −1.2 and −0.8 kb relative to the cap site of the human VEGF gene for hepatoma-specific enhancement of AFP promoter activity. A 47-bp sequence located 985–939 bp 5′ to the VEGF transcription initiation site mediates hypoxia-inducible expression that is directed by SV40 promoter (29). We constructed several hybrid promoters in which various deletions of the 0.4-kb fragment containing VEGF HRE or three copies of 24-bp VEGF HRE core sequence were directly linked to the 0.3-kb AFP promoter and examined those transcriptional activities. The deletions and the three copies of the HRE core sequence mediated only 30–70% less reporter gene expression than did the [HRE]AF promoter in hypoxic hepatoma cells (data not shown), probably because the HRE core sequence and the trans- and cis-acting elements existing in the AFP promotor were too close and because the transcription factors, such as HIF-1, HNF-1, and NF-1, might be unable to bind to these regions cooperatively.

Hypoxia-inducible expression of VEGF plays a central role in neovascularization, which is essential for tumor growth beyond 1–2 mm³. Several reports have shown that VEGF transcripts are overexpressed in HCC tissues as compared with noncancerous liver tissues, which express VEGF less intensely (30–32). Recently, other investigators have demonstrated by semiquantitative RT-PCR that there was no difference in VEGF expression between HCC and liver tissues with chronic HCV infection and that cirrhotic livers had significantly higher VEGF expression than did noncirrhotic livers (52, 53). Therefore, when the transfected genes are controlled by VEGF 5′-flanking full sequences, the expression could be induced in both HCC and noncancerous tissues. However, in the noncancerous tissues in which VEGF expression is detected, inflammatory cell infiltration is apparent, and the VEGF expression is stimulated not only by hypoxia but also by various cytokines and growth factors (32). Furthermore, Maxwell et al. (54) have described that HIF-1 or a closely related heterodimer involving HIF-1β/aryl hydrocarbon receptor nuclear translocator is the major mediator of VEGF expression within solid tumors, despite the multiple regulatory mechanisms operating on the VEGF gene. Therefore, the use of the VEGF hypoxia-inducible enhancer could allow us to exploit tumor hypoxia to obtain selective expression of genes and to achieve tumor-specific rather than tissue-specific targeting. Also, the [HRE]AF hybrid promoter could be useful for inducing targeted expression of therapeutic genes in HCCs that produce various amounts of AFP and for preventing their expression in hepatic stem cells that also express AFP.

The activity of the AFP promotor is originally weak, but we found that adding the VEGF hypoxia-inducible enhancer directly to the AFP promotor resulted in sufficient and specific expression of transfected genes, even in the solid tumors consisting of low-AFP-producing cells. In addition, transcriptional activation via the VEGF hypoxia-inducible enhancer, which contains HRE, is mediated by HIF-1, the activation of which occurs in the hypoxic cells of solid tumors. Thus, our construct can mediate efficient therapeutic gene expression selectively in human hepatoma cells, although in vivo studies using G418-unselected cells would be more relevant, and the effect of our construct on liver stem cells needed to be examined experimentally if the clinical application was being considered. This selective mediation would shed some light on strategies for gene therapy that targets HCC.

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