Therapeutic Efficacy of the Suicide Gene Driven by the Promoter of Vascular Endothelial Growth Factor Gene against Hypoxic Tumor Cells

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ABSTRACT

We examined whether herpes simplex virus thymidine kinase (HSV-TK) gene expression driven by the promoter of the vascular endothelial growth factor (VEGF) gene that is activated by hypoxia is effective in killing highly metastatic Lewis lung carcinoma A11 cells under hypoxic conditions. We isolated the promoter region encompassing the hypoxia response element (HRE) of the mouse VEGF gene. To assess the hypoxia responsiveness of the VEGF promoter, A11 cells were transiently transfected with luciferase reporter plasmids. Exposure of the transfectants to hypoxia resulted in a 2–3-fold induction of luciferase activity. Deletion of the HRE site abolished VEGF promoter activity under both normoxic and hypoxic conditions. We constructed a retroviral vector harboring the HSV-TK or green fluorescence protein (GFP) gene under the control of the VEGF promoter. A11 cells transfected with vector harboring the VEGF promoter fused to the HSV-TK gene [A11(HRE/TK) cells] were more sensitive to ganciclovir than cells transfected with the control vector harboring the VEGF promoter alone, and the sensitivity of the A11(HRE/TK) cells was increased by exposure to hypoxia followed by reoxygenation. Culturing A11 cells transfected with vector harboring the VEGF promoter fused to the GFP gene under hypoxic conditions resulted in an increase in the expression of GFP. Monitoring GFP expression and vascularity in the A11 transfectant tumors revealed up-regulation of GFP expression in poorly vascularized regions. Administration of ganciclovir to mice bearing s.c. tumors formed by A11(HRE/TK) cells resulted in regression of the tumors. These results suggest a possible application of the suicide gene driven by the VEGF promoter to cancer gene therapy that efficiently targets hypoxic tumor cells.

INTRODUCTION

Neovascularization is crucial for tumor growth and metastasis. Many angiogenic factors that regulate this process have been identified (1, 2). Among them is VEGF, which is one of the most effective mitogens specific for endothelial cells and has been implicated in the neovascularization of a variety of tumors (3–7). Most solid tumors have areas of low oxygen tension (hypoxia). Tumor cells under hypoxic conditions produce VEGF to stimulate angiogenesis (8). Recent studies have provided evidence that the expression of the VEGF gene is regulated by hypoxia at both the transcriptional and posttranscriptional levels (9). The transcriptional complex termed hypoxia-inducible factor-1 binds to the HRE site located in the 5’-flanking region of the VEGF gene and transactivates gene expression (10–12). The sequences in the 3’-untranslated region of VEGF mRNA contribute to the regulation of RNA stability under hypoxic conditions (12, 13). Hypoxia also triggers the expression of other genes such as erythropoietin and some glycolytic enzymes, and functional HRE is reported in these hypoxia-regulated genes (14, 15).

In tumor masses, besides chronic hypoxia, acute hypoxia is generated due to the opening and closing of blood vessels (16). Tumor cells in chronic hypoxia may become necrotic and eventually die. However, tumor cells in acute hypoxia can remain viable and are often resistant to chemotheraphy and radiation (17). Thus, hypoxia is an obstacle to cancer therapy. Supporting this, hypoxia is reported to be correlated with poor prognosis in cancer patients (18).

One of the major goals of cancer gene therapy is to increase tumor cell selectivity. To achieve this purpose, several attempts based on cell type-specific receptors aiming at targeted gene delivery and tissue-specific promoters aiming at heterologous gene expression in a specific organ have been made (19–23). In addition to these approaches, it is possible to take advantage of tumor hypoxia to obtain selective expression of heterologous genes. Dachs et al. (24) recently exploited the feasibility of this approach using the HRE-driven cytosine deaminase/ganciclovir system that this system could target hypoxic tumor cells in vitro. Because hypoxia occurs in many solid tumors, this approach would increase tumor cell selectivity and would be applicable to a wide range of tumors.

In the present study, we investigate whether expression of the HSV-TK gene driven by the VEGF promoter encompassing the HRE site followed by GCV administration is effective in killing highly metastatic Lewis lung carcinoma A11 cells under hypoxic conditions.

MATERIALS AND METHODS

Cells and Culture Conditions. Highly metastatic A11 cells derived from Lewis lung carcinoma (25, 26) were cultured in DMEM supplemented with heat-inactivated (56°C, 30 min) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were grown in a humidified atmosphere of 5% CO2 in air at 37°C. They were also cultured under hypoxic or anoxic conditions in a BBL Gaspak Pouch anaerobic system (Becton Dickinson, Cockeysville, MD), which reduces oxygen tension to less than 2% within 2 h of incubation.

RNA Extraction and Northern Blot Analysis. Total RNA was extracted with guanidinium thiocyanate (27) from A11 cells cultured under normoxic or hypoxic conditions. Total RNA (20 μg) was electrophoresed on a 1% agarose gel containing formaldehyde and transferred to nylon filters. Blots were hybridized with a 32P-labeled mouse VEGF cDNA probe, which was prepared by the random primer method (27). VEGF cDNA was obtained by reverse transcription-PCR using total RNA isolated from A11 cells. The sense and antisense primers were 5’-AGAGAGGGAGCGCGAGCCG-3’ and 5’-GGAATTCGCTTCGCGGAGTCT-3’, respectively. The sequence of the PCR product was verified by DNA sequencing and found to encode the reported mouse VEGF (GenBank accession number S38100). Filters were finally washed at 50°C in 30 mM NaCl, 3 mM sodium citrate, and 0.1% SDS.

Cloning and Sequencing of the Mouse VEGF Promoter Region. The VEGF promoter region was amplified by PCR using genomic DNA isolated from mouse liver, oligonucleotide primers synthesized on the basis of the published DNA sequence (GenBank accession number U41383; Ref. 28), and LA Taq DNA polymerase (TaKaRa Biomedicals, Osaka, Japan). The sense and antisense primers were 5’-GGTTATAGATGTACCGGACCTTACG-3’ and 3’-ATGACCTTCTTGCTTCAGTGTA-3’, respectively. The PCR conditions were 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 68°C for 3 min, and 72°C for 7 min. The PCR product, which contained the 5’-flanking sequence encompassing the putative HRE site, the transcription

Received 7/26/99; accepted 4/14/00.

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The abbreviations used are: VEGF, vascular endothelial growth factor; HSV-TK, herpes simplex virus thymidine kinase; HRE, hypoxia response element; GFP, green fluorescence protein; GCV, ganciclovir; DMSO, Dibucaine’s PBS; LTR, long terminal repeat; TRITC, tetramethylrhodamine isothiocyanate; SV40ApA, SV40 polyadenylation signal.

CANCER RESEARCH 60, 2936 –2941, June 1, 2000
GROWTH INHIBITION ASSAY.

Cells were exposed to different concentrations of GCV for 5 days. Alternatively, cells were subjected to hypoxia for 16 h followed by exposure to GCV for 24 h in air, and the cell number was determined 2 days after the treatment.

In Vivo Experiments. Cells (2.5 × 10^6) retrovirally transduced with LV(HRE)TK or LV(HRE) were s.c. injected into 6-week-old female C57BL/6 mice. Ten days after the inoculation, GCV diluted in DPBS was i.p. injected at a concentration of 30 mg/kg twice daily at 8-h intervals for 5 days. DPBS alone was injected into control mice. Tumor growth was monitored by caliper measurement of two diameters at right angles, and the tumor mass was estimated from the equation volume = 0.5 × a × b^2, where a and b are the larger and smaller diameters, respectively (29).

RESULTS

Induction of VEGF mRNA Expression in A11 Cells in Response to Hypoxia. To examine whether A11 cells respond to hypoxia and express VEGF, the cells were cultured under hypoxic conditions for up to 24 h, and the expression of VEGF mRNA was examined by Northern blot analysis. As shown in Fig. 1, the amount of VEGF mRNA dramatically increased after the cells were subjected to hypoxia. Thus, A11 cells responded well to low oxygen tension.

Responsiveness of Mouse VEGF Promoter Sequences to Hypoxia in A11 Cells. We isolated the mouse VEGF promoter region with or without the HRE site and constructed luciferase reporter plasmids pGLV(HRE)Luc or pGLV(ΔHRE)Luc, respectively (Fig. 2). A11 cells were transiently transfected with the reporter plasmids and cultured for 48 h. During the last 18 h, the cells were subjected to hypoxia, and cell lysates were assayed for luciferase activity. As shown in Fig. 3, the VEGF promoter sequence produced an increase in luciferase activity under normoxic conditions. Culturing the cells under hypoxic conditions resulted in a 2–3-fold enhancement of luciferase activity compared with normoxic conditions. Deletion of the HRE site from the promoter sequence abolished luciferase activity under both normoxic and hypoxic conditions (Fig. 3).

Transfection of Retroviral Vectors Harboring the HSV-TK Gene under the Control of VEGF Promoter and Sensitivity to GCV. A11 cells were transfected with LV(HRE)TK or LV(ΔHRE)/TK, retroviral vectors harboring the HSV-TK gene under control of the VEGF promoter sequence with or without the HRE site, or LV(HRE), a retroviral vector harboring the VEGF promoter sequence alone (Fig. 2), and after selection with G418, they were established as A11(HRE/TK), A11(ΔHRE/TK), or A11(HER) cells.

Incubation time (hr)
0 3 6 9 12

Fig. 1. Induction of VEGF mRNA in A11 cells in response to hypoxia. A11 cells were cultured for the indicated times under hypoxic conditions. Total RNA was isolated from the cells and subjected to Northern blot analysis. Blots were hybridized with ^3P-labeled VEGF cDNA. Ethidium bromide staining of the gel is also shown.

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respectively. The susceptibility of these cell lines to the toxicity of GCV is shown in Fig. 4. Even under normoxic conditions, A11(HRE/TK) cells were approximately 200-fold more sensitive to GCV than the control A11(HRE) cells (Fig. 4A). However, A11(DHRE/TK) cells showed a sensitivity to GCV similar to that of the control cells (Fig. 4A).

To test whether the VEGF promoter-regulated HSV-TK expression is able to sensitize hypoxic cells to GCV, A11(HRE) and A11(HRE/TK) cells were exposed to hypoxia for 16 h followed by exposure to GCV for 24 h in air. As expected, A11(HRE/TK) cells, but not A11(HRE) cells, became more sensitive to GCV after exposure to hypoxia (Fig. 5, A and B). A slight increase in GFP expression was detected after culturing the cells under hypoxic conditions (data not shown), and a more pronounced expression was observed after exposure to hypoxia followed by reoxygenation (Fig. 6B). A flow cytometric analysis also confirmed these results (data not shown).

To investigate whether GFP expression is also observed in tumor cells in vivo, we monitored GFP expression in combination with assessment of vascularity, which was examined by CD31 immunostaining in cryosections prepared from s.c. tumors formed by A11(HRE/GFP) cells. The results showed that up-regulation of the GFP expression was observed in poorly vascularized regions (Fig. 7, A and B) compared with well-vascularized regions (Fig. 7, C and D).

**Luciferase reporter plasmid**

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<tr>
<th>pGLV(HRE)Luc</th>
<th>HRE</th>
<th>VEGFpro</th>
<th>Luc</th>
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<tr>
<td>pGLV(ΔHRE)Luc</td>
<td>ΔHRE</td>
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**Retrovirus vector**

<table>
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<tr>
<th>LV(HRE)</th>
<th>LTR</th>
<th>SV40pA</th>
<th>VEGFpro</th>
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<tr>
<td>LV(HRE)TK</td>
<td>LTR</td>
<td>SV40pA</td>
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<td>LV(ΔHRE)TK</td>
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<td>LV(HRE)GFP</td>
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Fig. 2. Schematic representation of the luciferase reporter and retroviral constructs. VEGFpro, VEGF promoter region; Luc, luciferase gene. VEGFpro, HSV-TK, GFP, and SV40pA were inserted into retroviral vector LXSN(BA) in a reverse orientation of LTR.

**Fig. 3.** Functional analysis of the mouse VEGF promoter in A11 cells. Luciferase activities were obtained from A11 cells that were transiently transfected with VEGF promoter-luciferase constructs. pGLV(HRE)Luc or pGLV(ΔHRE)Luc is the construct in which the VEGF promoter with or without the HRE site is fused to the luciferase gene, respectively. A11 cells were exposed to normoxia or hypoxia for 18 h. All luciferase activities were normalized for transfection efficiency, and background luciferase activities obtained from A11 cells transfected with a promoterless luciferase gene pGL2-basic were subtracted from this value. Luciferase activity is expressed in arbitrary units.

Fig. 4. Sensitivity of A11 cells to GCV. A, A11(HRE) (○), A11(HRE/TK) (●), and A11(ΔHRE/TK) (□) cells were cultured with various concentrations of GCV for 5 days. B, VE8 (○), TK17 (●), and TK18 cells (□) were cultured with various concentrations of GCV for 5 days.

Fig. 5. Introduction of a Retroviral Vector Harboring the GFP Gene under the Control of the VEGF Promoter and GFP Expression. We constructed retroviral vector LV(HRE/GFP), which harbors the GFP gene under the control of the VEGF promoter sequence (Fig. 2). A11 cells were transduced with the retroviral construct, and a stable transfectant termed A11(HRE/GFP) was obtained after selection with G418. The cells were exposed to hypoxia for 16 h followed by reoxygenation for 3 h and examined for GFP expression. A basal level of GFP expression was observed in normoxic A11(HRE/GFP) cells (Fig. 5A). A slight increase in GFP expression was detected after culturing the cells under hypoxic conditions (data not shown), and a more pronounced expression was observed after exposure to hypoxia followed by reoxygenation (Fig. 6B). A flow cytometric analysis also confirmed these results (data not shown).
Therapeutic Efficacy of GCV Administration. A11, VE8, TK17, or TK18 cells were s.c. injected into age-matched C57BL/6 mice, and 10 days after the inoculation, GCV (30 mg/kg) was administered i.p. to the mice. TK17 cells grew more slowly than A11, VE8, and TK18 cells in vivo. As shown in Fig. 8, in the TK17 and TK18 cells, GCV administration resulted in regression of the tumors. The growth of VE8 cells was not influenced by GCV, and was comparable to that of A11 cells.

**DISCUSSION**

In the present study, we assessed the regulatory activity of the mouse VEGF promoter sequence in A11 cells that expressed a high level of VEGF mRNA when cultured under hypoxic conditions. VEGF promoter sequence fused to the reporter consistently produced a severalfold increase in luciferase activity in response to hypoxia. Thus, the VEGF promoter was suggested to be activated in hypoxic tumor cells. However, the activation of the VEGF promoter by hypoxia was about 2–3-fold, whereas the expression of VEGF mRNA in situ was dramatically increased. The difference may be due to the fact that the hypoxia-induced regulation of VEGF gene expression occurs at the levels of both mRNA transcription and stability (9, 12, 13). Interestingly, deletion of the HRE site abolished luciferase activity in both normoxic and hypoxic A11 cells, indicating that the reporter gene expression was HRE dependent. This result is consistent with reports that deletion of the HRE site from the human and mouse VEGF genes abolished hypoxia inducibility in C6 glioma cells (31) and the perinecrotic palisading cells in GS9L gliomas, respectively (13). However, there are reports that deletion of the HRE site from mouse VEGF genes only weakened reporter gene expression in C6 glioma cells (28) and that rat and human VEGF genes with mutations or deletions of the HRE consensus still retain residual hypoxia inducibility in PC12 and Hep3B cells, respectively (10, 11). Where the difference comes from is presently unclear. However, it is possible that a complex cell type-specific regulation of VEGF gene expression under hypoxic conditions may cause the difference.

To determine whether VEGF promoter-driven gene expression is applicable to cancer gene therapy, we constructed retroviral vector LV(HRE)/TK. In this construct, the VEGF promoter sequence and the HSV-TK gene were inserted in the opposite orientation to the direction of expression of the viral mRNA driven from the 5'-LTR to minimize nonspecific expression of HSV-TK. A similar ap-
approach was successfully used to express a heterologous gene under the control of cell- or tissue-specific promoters in a retroviral vector construct (24, 25). A11 cells were transfected with LV(HRE)TK, and their sensitivity to GCV was examined. Under normoxic conditions, the transfected cells were more susceptible to GCV than the control cells. Deletion of the HRE site from the VEGF promoter sequences abolished the sensitivity to GCV. Thus, in agreement with the luciferase assays, the VEGF promoter activity in the retroviral construct is also HRE dependent. Importantly, the LV(HRE)TK-transfected A11 cells became more susceptible to GCV after exposure to hypoxia followed by reoxygenation.

We next addressed whether VEGF promoter-driven GFP expression is up-regulated in solid tumors, especially in hypoxic areas. In vitro experiments demonstrated that exposure of cells transfected with retroviral vector LV(HRE)GFP to hypoxia resulted in an increase in GFP expression. We then s.c. injected the transfected cells into syngeneic mice and monitored GFP expression in cryosections prepared from the solid tumors formed by the cells. The results showed that the level of GFP expression was up-regulated in poorly vascularized regions compared with well-vascularized regions. Although poor vascularization cannot necessarily be taken as a reflection of low oxygen supply, these results strongly suggest that GFP expression was increased in hypoxic areas in the solid tumors.

To investigate the therapeutic efficacy of the VEGF promoter HSV-TK approach, we s.c. injected VE8, TK17, and TK18 cells into syngeneic mice, and the mice were given GCV. Both TK17 and TK18 cells were more susceptible to GCV than VE18 cells and became more sensitive after exposure to hypoxia in vitro. Administration of GCV to mice bearing fast-growing TK18 cells and slow-growing TK17 cells resulted in regression of the tumors. Thus, HSV-TK gene expression under the control of the VEGF promoter followed by GCV administration was effective in suppressing tumor growth in vivo. Presently, however, there is no experimental evidence that GCV is more effective in vivo against tumor cells in hypoxic microenvironments than in normoxic microenvironments. Nevertheless, on the basis of the results of luciferase reporter assays and GFP expression in tumors, it can be assumed that the growth of normoxic tumor cells is suppressed by GCV due to the constitutive expression of HSV-TK and that the growth of hypoxic tumor cells is more strongly suppressed by GCV. Bystander effect should augment the effect of GCV,
as observed in other tumor models (32–34). Summation of these effects may lead to regression of the tumors.

In the present study, we did not examine the effect of the VEGF promoter/HSV-TK approach on tumor cells in micrometastases. However, it is known that oxygen tension in micrometastases is low enough to stimulate neoangiogenesis (35). Furthermore, recent studies indicate that highly metastatic tumor cells overexpress VEGF (36–38). We have also found that A11 cells produced a much greater amount of VEGF than low metastatic counterpart P29 cells (25, 29) in response to hypoxia.4 Collectively, these results suggest that the approach should be effective in treating secondary metastatic disease.

A major obstacle to the gene therapy approach described here is gene and drug delivery to acute hypoxic areas in tumors. However, dynamic changes in tumor perfusion due to the closing and opening of blood vessels (16) might make repeated infusion appropriate.

In conclusion, our experiments demonstrated the feasibility of the application of the HSV-TK gene under control of the VEGF promoter against normoxic tumor cells as well as hypoxic tumor cells that are often resistant to chemotherapy and radiation. Although we used the authentic mouse VEGF promoter, it was previously shown that inclusion of multiple copies of the HRE site enhanced the hypoxic response (24, 39). The HRE site of human origin was more responsive to hypoxia than that of mouse origin (39). Furthermore, inclusion of 3′-untranslated sequences from the VEGF gene downstream of a lacZ reporter gene resulted in increased β-galactosidase staining in the perinecrotic palisading cells (13). These improvements undoubtedly increase hypoxia responsiveness and hypoxic cell selectivity of the gene therapy approach described here.

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*Cancer Res* 2000;60:2936-2941.