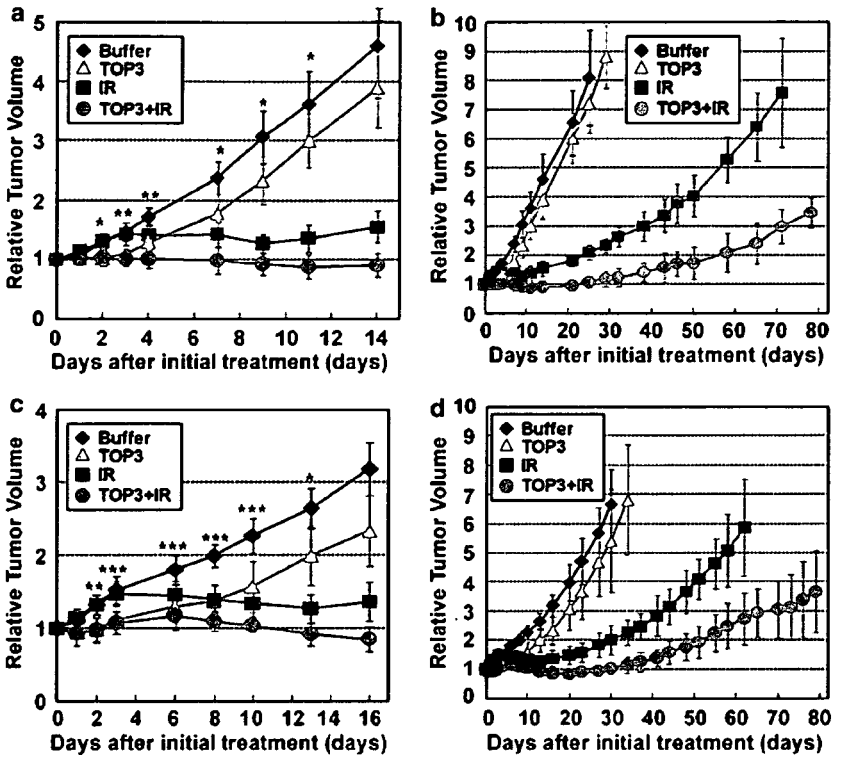

Figure 5 Antitumor effect induced by a combination of TAT-ODD-procaspase-3 (TOP3) and ionizing radiation (IR). Tumor-bearing mice ($n=10$ for each group) with HeLa/5HRE-Luc or CFPAC-1 xenografts in the right hind leg were treated as described in the legends to Figures 3 and 4; The mice of the TOP3 and the TOP3 and IR groups were treated with TOP3 on day 0, 1 and 2, and the mice of the IR and the TOP3 and IR groups were irradiated with 10 Gy on day 2. Tumor volume of HeLa/5HRE-Luc (**a** and **b**) and CFPAC-1 (**c** and **d**) xenografts treated as above were measured until day 78 and day 79, respectively. The relative tumor volume (the ratio of tumor volumes on each day to the corresponding ones on day 0) of HeLa/5HRE-Luc until day 14 (**a**) and day 78 (**b**) and the one of CFPAC-1 xenografts until day 16 (**c**) and day 79 (**d**) are presented in the graphs. The results are the mean of 10 independent tumors±s.d. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ (none vs TOP3).

In this study, we treated tumors with the combination of IR and a hypoxia-targeting drug, TOP3. In monotherapy experiments, TOP3 temporarily suppressed tumor growth, but was not effective on long-term tumor growth inhibition (Figures 5b and d). Inhibition of HIF-1 by TOP3 was also temporary (Figures 3a and b). The combined treatment, however, resulted in significantly enhanced long-term suppression of tumor growth compared to either treatment alone (Figures 5b and d). Our results suggest that the enhanced effect of the combined treatment on long-term growth suppression (Figures 5b and d) was not simply due to an additive effect of TOP3 and IR on the tumor cells. We found that the microvessel density in the tumors treated with the combination was significantly reduced compared to either treatment alone in both short- and long-term-treated tumors (Figure 6). This result suggests that the enhanced growth suppression of the combination was due in part to the anti-angiogenic efficacy of the combined treatment. These results further indicate that the intratumoral HIF-1 activity is essential for the short-term restoration of damaged microvessels and long-term regrowth of irradiated tumors. Conversely, these results confirmed that hypoxia and its associated HIF-1 activity limit the efficacy of radiotherapy.

Recently, Moeller *et al.* (2004) demonstrated that irradiation of tumor xenografts with 5, 10 and 15 Gy induced HIF-1 activity, leading to the increased expression of VEGF and basic fibroblast growth factor, which act to prevent radiation-induced endothelial cell death. They also revealed that HIF-1 activity peaked at 48 h after radiation and provide evidence that radiation-induced reoxygenation of hypoxic tumor cells results in the production of reactive oxygen species (ROS) that induce HIF-1 activity. Our results are consistent with theirs: HIF-1 activity peaked at 2 days after IR (Figure 2) and suppression of HIF-1 activity resulted in reduced angiogenesis (Figures 6a and b) and significant enhancement of the tumor growth suppression by IR (Figures 5b and d).

Small molecules targeting HIF-1 have been developed which include 2-methoxyestradiol, 17-Allylamino-17-demethoxygeldanamycin, camptothecin, topotecan, pleurotin and YC-1. They inhibited HIF-1 activity and delayed tumor growth (Rapisarda *et al.*, 2002; de Candia *et al.*, 2003; Mabjeesh *et al.*, 2003; Semenza, 2003; Welsh *et al.*, 2003; Yeo *et al.*, 2003; Moeller *et al.*, 2004). In addition, novel inhibitors of signal transduction pathways were also observed to decrease the level of HIF-1α protein with resulting antiangiogenic effects

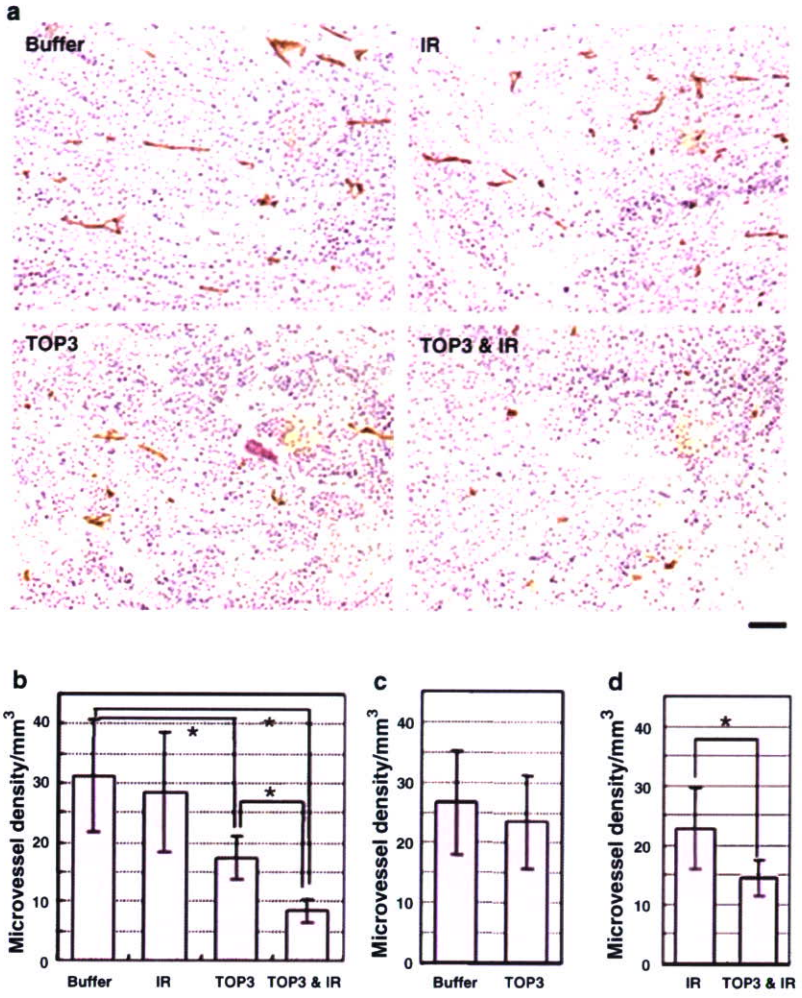

Figure 6 Inhibitory effect on tumor neovascularization induced by a combination of TAT-ODD-procaspase-3 (TOP3) and ionizing radiation (IR). (**a**) Tumor-bearing mice with HeLa/5HRE-Luc xenografts in the right hind leg were treated with buffer, TOP3, 10 Gy (IR) and TOP3 plus 10 Gy (TOP3 and IR) as described in the legend to Figure 5. The xenografts were surgically excised on day 14 and processed for immunohistochemical analysis with anti-CD31 antibody. A representative image of the tumor section in each treatment group is presented. Bar = 100 μm. (**b**) The number of the CD31-positive blood vessels per square millimeter (microvessel density/mm^3) in the section of the tumor on day 14 (Figure 6a) was counted under a microscope and the mean of 10 areas in three to four independent tumors for each treatment group is shown in the graph. (**c**) The xenografts from untreated (buffer) and TOP3-treated (TOP3) mice in Figure 5b were surgically excised on day 30 and analysed for their microvessel densities as Figure 6b legend. (**d**) The xenografts treated with 10 Gy (IR) or with both TOP3 and 10 Gy (TOP3 and IR) in Figure 5a were surgically excised on day 40 and analysed for microvessel density as described above. $^*P<0.01$.

(Semenza, 2003). The mechanism of action of these small-molecule drugs has yet to be elucidated, and it is not known whether or not tumors develop resistance to them.

Our approach differs from small-molecule targeting of HIF-1. The small-molecules reduce HIF-1 activity, but do not kill the hypoxic cells. Therefore, these cells remain hypoxic and resistant to radiotherapy. In contrast, TOP3 kills HIF-1-active cells, which reside in the hypoxic region of the tumor and those arising during reoxygenation shortly after irradiation. Our approach reduces the problem of radioresistance of hypoxic cells and tumor regrowth stimulated by HIF-1-induced neovascularization.

HIF-1 is not the only hypoxia-regulating transcription factor in the cell. Other hypoxia-responsive factors such as HIF-2 are induced in hypoxic tumors, which are unlikely to be influenced by small-molecule HIF-1 inhibitors. In contrast, TOP3 kills all the hypoxic cells and therefore is a wider spectrum drug against hypoxic tumors. An additional possible advantage of TOP3 over small HIF-1 inhibitors is that its mechanism of action is clearly understood (Harada *et al*., 2006). TOP3 is activated only in HIF-1-active cells. If the tumors were to develop resistance to this newly developed drug, they would have to accumulate mutations, which inactivate the HIF-1 and prevent stabilization of HIF-1 in

hypoxia. This would result in the inability of tumors to induce neovascularization after therapy, which would prevent tumor regrowth.

In summary, we found in this study that TOP3 effectively eradicated HIF-1-active cells and as a consequence its application resulted in short-term suppression of angiogenesis in xenograft. This short-term anti-angiogenesis when combined with radiotherapy leads to long-term suppression of microvessels and total reduction of the hypoxic fraction in tumor xenograft.

Although we have been confirmed that stabilization itself does not endow TOP3 with cytocidal activity (Harada *et al.*, 2006) and that TOP3 has not caused obvious side effects in animal experiments (Harada *et al.*, 2002, 2005), as for the clinical application of a newly developed drug, the safety issue is the major concern. Since TOP3 eradicates HIF-1-active hypoxic cells, which are at the concentration far below their optimal oxygen concentration, its use in cancer patients with concomitant ischemic diseases may potentially pose a problem. In the case of ischemia however, the hypoxic condition is likely to be transient instead of permanent as in the case of tumors, and we still have no knowledge on how TOP3 affects such cells in a patient. In order to answer this important question, we have recently developed imaging probes, which detect such transient ischemia in a body by combining PTD, ODD and an imaging function domain (Tanaka *et al.*, in preparation). With these probes in our hand, we are currently assessing the effect of TOP3 on transient ischemia in experimental systems.

Materials and methods

Cell culture and hypoxic treatment in vitro
The CFPAC-1 human pancreatic cancer cell line and HeLa human cervical epithelial adenocarcinoma cell line were purchased from the American Type Culture Collection. HeLa/EF-Luc and HeLa/5HRE-Luc that carry the luciferase reporter under control of the HIF promoter, were isolated as described previously (Harada *et al.*, 2005). CFPAC-1 and HeLa cells were maintained at 37°C in 10% fetal bovine serum (FBS)–Iscove's modified Dulbecco's medium (Life Technologies, Inc., Rockville, MD, USA) and 5% FBS–Dulbecco's modified Eagle's medium (Nacalai Tesque, Kyoto, Japan), respectively.

Formulation of TOP3 fusion protein
TOP3 was dissolved in 10 mM Tris-HCl buffer (pH 8.0) at a final concentration of 15 μg/ml if not otherwise indicated.

Tumor model
HeLa/5HRE-Luc or CFPAC-1 cells were subcutaneously inoculated at 1×10^6 cells/100 μl of phosphate-buffered saline (PBS) into the right hind leg of 6-weeks-old female nude mice (BALB/c nu/nu; Japan SLC Inc., Hamamatsu, Japan). Day 0 was designated when tumors grew to approximately 200 mm^3 10 days after cell inoculation. These mice were then i.p. injected with TOP3 (20 mg/kg) on day 0, 1 and 2. Long-term monitoring of tumor growth was done by measuring the size of the tumors with calipers. Tumor volume was calculated as $0.5LW^2$.

Real-time monitoring of luciferase activity in vivo
Tumor-bearing mice were intravenously injected with 100 μl D-luciferin solution (10 mg/ml in PBS; Promega Corp., Madison, WI, USA). Exactly 3 min later, the mice were put in an IVIS-200 *in vivo* photon-counting device (Xenogen Corp., Alameda, CA, USA). The pseudo images from the photon counts were constructed by Living Image 2.50 – Igor Pro 4.09A software (Xenogen Corp.). Each image was quantified as photons/s and normalized against the day 0 photon counts. Each experimental group contained five mice.

Radiation treatment
Tumor-bearing mice were locally irradiated with 10 Gy of ^{137}Cs γ-ray using a collimator set at the tumor site with a Gammacell 40 Exactor (MDS Nordion International Inc., Ontario, Canada). The control tumor-bearing mice were sham irradiated.

Immunohistochemical analysis
Pimonidazole hydrochloride (60 mg/kg; Natural Pharmacia International Inc., Belmont, MA, USA) was i.p. injected into tumor-bearing mice at 90 min before surgical excision of solid tumors. The excised solid tumors were fixed in 10% formalin neutral buffer solution (pH 7.4; Wako Pure Chemical Industries Inc., Osaka, Japan) and embedded in paraffin. To detect pimonidazole-binding and luciferase proteins, serial sections prepared from the paraffin-embedded sections were treated with anti-pimonidazole (Natural Pharmacia International, Inc.) and anti-luciferase (Promega, Madison, WI, USA) antibodies, respectively. To detect CD31, surgically excised solid tumors were frozen in liquid N_2 and embedded in optimal cutting temperature (OCT). Sections prepared from the OCT-embedded tumor were treated with anti-CD31 antibody (BD Bioscience Pharmingen, San Diego, CA, USA). Staining was done with the indirect immunoperoxidase detection method (DakoCytomation, Carpinteria, CA, USA). Counter staining was done with hematoxylin. Paraffin-embedded serial sections were also stained with hematoxylin and eosin (H&E). Microvessel density was measured by counting the number of vessels (regions stained with anti-CD31 antibody) per square millimeter in each image.

Statistical analysis
Data are expressed as means ± s.d. Statistical significance of differences was determined by the paired two-tailed Student's *t*-test. Differences were considered statistically significant for $P<0.05$.

Abbreviations

HIF-1, hypoxia-inducible factor-1; PTD, protein-transduction domain.

Acknowledgements

We are grateful to Dr Ohtsura Niwa for extensive discussion; Akiyo Morinibu, Emi Nishimoto and Naoko Harada for skilled technical assistance. This work was supported in part by grant-in-aid for Scientific Research on Priority Areas, Cancer, from the Ministry of Education, Culture, Sports, Science and Technology, and by a grant-in-aid for the second- and

third-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare, Japan. This study is a part of joint research, which is focusing on the development of the basis of technology for establishing COE for nano-medicine, carried out through Kyoto City Collaboration of Regional Entities for Advancing Technology Excellence (CREATE) assigned by Japan Science and Technology Agency (JST).

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Biochemical and Biophysical Research Communications 360 (2007) 791–796
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The combination of hypoxia-response enhancers and an oxygen-dependent proteolytic motif enables real-time imaging of absolute HIF-1 activity in tumor xenografts ☆

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Received 20 June 2007
Available online 5 July 2007

Abstract

The transcriptional activity of hypoxia-inducible factor-1 (HIF-1) is associated with tumor malignancies; therefore, it is important to comprehend its dynamism in solid tumors. However, a molecular imaging strategy to accurately access it remains to be developed. We constructed here a novel HIF-1-dependent reporter gene, *5HREp-ODD-luc*, in which 5 copies of the hypoxia-response element (5HRE) enhance expression of the oxygen-dependent degradation (ODD) domain and luciferase (luc) fusion under hypoxia. Because the ODD domain caused the oxygen-dependent degradation of the ODD-Luc protein, the novel reporter gene showed little leak of luminescence under normoxia. Such a property caused an increase of the hypoxia-responsiveness up to about 4.7×10^4-fold. Moreover, the ODD domain caused rapid degradation of the ODD-Luc protein under normoxia, the luminescence reflected the dynamism of HIF-1 activity in real-time. The superiority of the novel reporter gene will surely accelerate analysis of the intratumoral HIF-1 activity during tumor progression and cancer treatments.

Keywords: Hypoxia; Hypoxia-inducible factor-1 (HIF-1); Hypoxia-response element (HRE); Oxygen-dependent degradation domain (ODD domain); Optical imaging

The exponential proliferation and aberrantly accelerated metabolism of cancer cells result in an imbalance of "oxygen-supply" and "oxygen-consumption" in solid tumors. Such disequilibrium is a major causative factor of tumor hypoxia, a unique microenvironment in locally advanced solid tumors [1,2]. Hypoxia-inducible factor-1 (HIF-1) plays pivotal roles in the cellular adaptive response to such a severe microenvironment [3]. HIF-1 is a heterodimeric transcription factor composed of α- (HIF-1α) and β-subunits (HIF-1β), the activity of which is mainly dependent on the stability of the former [4]. Under normoxic conditions, the oxygen-dependent degradation (ODD) domain of HIF-1α is modified by PHDs and pVHL, resulting in the degradation of HIF-1α protein with a half-life of about 5–8 min [5,6]. On the other hand, HIF-1α is stabilized

Abbreviations: HIF-1, hypoxia-inducible factor-1; HRE, hypoxia-response element; ODD, oxygen-dependent degradation; luc, luciferase; 5HREp, 5 copies of HRE promoter; GFP, green fluorescent protein.

☆ Ethics of Experiment: All of our animal experiments were approved by the Animal Research Committee of Kyoto University and carried out in accordance with its guidelines.

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doi:10.1016/j.bbrc.2007.06.149

under hypoxic conditions and interacts with HIF-1β [4]. The resultant HIF-1 binds to its cognate DNA sequence, hypoxia-responsive element (HRE), and induces the expression of various genes [3,7,8]. The HIF-1-dependent factors are known to induce the malignant phenotypes of cancer cells and result in resistance to current cancer therapies [9,10]. Therefore, it is important to analyze the dynamism of intratumoral HIF-1 activity during carcinogenesis, tumorigenesis, and current antitumor therapies.

To date, HIF-1α-expression in tumor xenografts has been mainly analyzed by immunohistochemistry. However, with this method, it is impossible to follow up the changes of expression sequentially in individual living animals. Therefore, extensive efforts have been devoted to develop an alternative method; that is a molecular imaging strategy by the use of a HIF-1-dependent reporter gene [11]. Many groups have reported that a tandem repeat of HREs enhances the transcription of reporter genes under hypoxic conditions [11]. Above all, the 5HRE promoter (5HREp), in which 5 copies of the HRE enhance transcription from a cytomegalovirus minimal promoter, enhances by more than 500-fold luciferase (luc) or green fluorescent protein (GFP) expression under the control of HIF-1 *in vitro* [11–14]. With this reporter gene system (*5HREp-luc* or *5HREp-GFP* gene), optical imaging of intratumoral HIF-1 activity was accomplished in tumor xenograft models [14,15]. However, this reporter system still have some problems to improve; (1) it shows a certain amount of unwanted base gene expression even under normoxic conditions (leakage), and (2) excess stability of the reporter proteins makes it impossible to sense the dynamism (especially rapid degradation) of both HIF-1α protein and HIF-1 activity (carry-over) [14].

In the present study, we constructed a novel HIF-1-dependent reporter gene, *5HREp-ODD-luc*, in which the ODD-Luc fusion protein is expressed under the regulation of the 5HRE promoter. Because the ODD domain is fully responsible for the oxygen-dependent degradation of HIF-1α protein, the stability of the ODD-Luc fusion protein correlates to that of HIF-1α. Thus, the novel reporter gene enabled us to image absolute HIF-1 activity in real-time.

Materials and methods

Cell culture. The human cervical epithelial adenocarcinoma cell line, HeLa, was purchased from American Type Culture Collection and maintained in 10% FBS-Dulbecco's modified Eagle's medium. For normoxic cultures, cells were incubated in a well-humidified incubator with 5% CO_2 and 95% air at 37 °C. For hypoxic cultures (<0.02% O_2), cells were incubated in a Bactron Anaerobic Chamber, BACLITE-2 (Sheldon Manufacturing Inc., Cornelius, OR).

Plasmid DNA. The plasmid pEF/Luc constitutively expressing the luciferase gene and pEF/5HRE-Luc harboring *5HREp-luc* were constructed as described previously [15]. To construct the plasmid p5HRE-ODD-Luc encoding the *5HREp-ODD-luc* reporter gene, cDNA for a part of the ODD domain (548–603 a.a. of HIF-1α) was amplified from pCH/3-0 by PCR using as primers; 5′-AACCATGGCGCCTAAGAAGAA-GAGGAAG-3′ and 5′-AACCATGGTCTGCTGGAATACTGTA-ACTG-3′. The PCR product was digested with NcoI and inserted into the NcoI recognition site of pEF/5HRE-Luc [15].

Isolation of HeLa/5HRE-ODD-Luc cell. HeLa cells were transfected with p5HRE-ODD-luc by the calcium phosphate method [16] and cultured for 10 days in culture medium containing 400 μg/ml of G418 (Nacalai Tesque, Kyoto, Japan). Antibiotic-resistant colonies were isolated and established as clones, and a representative clone was used in the present study. HeLa/EF-Luc cells, which constitutively express luciferase protein, and HeLa/5HRE-Luc cells, which express the luciferase gene under the control of the 5HRE promoter, were established as described previously [15].

Luciferase assay and Western blotting. Cells were seeded in 24-well plates (2×10^4 cells/well) and subjected to various treatments in each experiment. The cells were washed with PBS and lysed with 100 μl of Passive Lysis Buffer (Promega, Madison, WI) for the luciferase assay or with 100 μl of 1× SDS–PAGE loading buffer for the Western blot analysis. The luciferase assay was performed by using Luciferase Assay Reagent (Promega) according to the manufacturer's instructions. The Western blot analyses for HIF-1α and Luciferase protein were performed by using anti-HIF-1α monoclonal antibody (BD Bioscience, San Diego, CA) and anti-luciferase polyclonal antibody (Promega), respectively, as described previously [15].

Real-time imaging of luciferase activity in tumor xenografts. Suspensions of HeLa/EF-Luc and either HeLa/5HRE-Luc or HeLa/5HRE-ODD-Luc cells (1×10^6/100 μl of PBS) were subcutaneously injected into the left and right legs of 6-week-old nude mice (BALB/c nu/nu, SLC, Hamamatsu, Japan), respectively. The tumor-bearing mice were used for each experiment 10 days after the implantation. For the *in vivo* imaging experiment, the tumor-bearing mice were intravenously injected with 100 μl of D-luciferin solution (10 mg/ml in PBS, Promega), and subjected to imaging with an IVIS-200 *in vivo* imaging system (Xenogen, Alameda, CA), as described previously [15]. Bioluminescent images were acquired just 3 min after the luciferin injection if not indicated.

Results and discussion

Construction of 5HREp-ODD-luc reporter gene

The conventional *5HREp-luc* reporter gene, which expresses the luciferase gene under the control of a HIF-1-dependent 5HRE promoter (Fig. 1), shows robust bioluminescence in response to hypoxic stimuli [13,15]. However, the reporter gene possesses two disadvantages (leakage and carry-over) for *in vivo* real-time imaging (see Introduction for details). To overcome these problems, a novel reporter gene, *5HREp-ODD-luc*, was constructed,

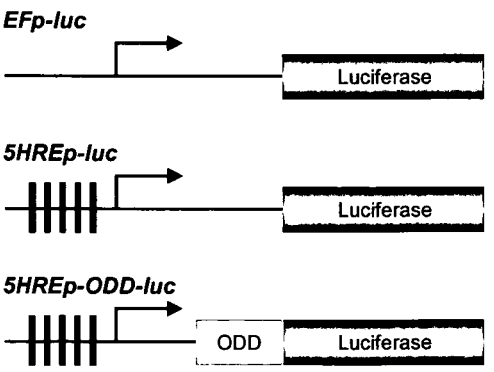

Fig. 1. Schematic constructs of the *EFp-luc* gene, *5HREp-luc* gene, and *5HREp-ODD-luc* gene.

in which a part of the ODD domain (548–603 a.a. of HIF-1α) was fused to the luciferase gene, and the fusion gene was expressed under the regulation of the HIF-1-dependent 5HRE promoter (Fig. 1). Because the ODD domain is fully responsible for the oxygen-dependent degradation of HIF-1α protein with a half-life of 5–8 min [6], it was estimated that the bioluminescence from the ODD-Luc fusion protein would have an identical half-life with HIF-1 and correlate to the absolute HIF-1 activity in real-time. In the following, we describe the superiority of the *5HREp-ODD-luc* reporter gene in detail.

5HREp-ODD-luc reporter gene senses increases of HIF-1 activity in real-time

First, we examined whether the *5HREp-ODD-luc* reporter gene senses the accumulation of HIF-1α protein and the resultant increase of HIF-1 activity under hypoxic conditions. HeLa cells stably transfected with the *5HREp-ODD-luc* gene, HeLa/5HRE-ODD-Luc cells, were cultured under hypoxic conditions, and the correlation between HIF-1α expression and the expression or bioluminescence of ODD-Luc were analyzed by Western blotting and luciferase assay. During hypoxic treatment, the bioluminescence from ODD-Luc protein gradually increased over time and reached more than 6.0×10^5 relative luciferase units (RLU) (Fig. 2A). Such hypoxia-dependent bioluminescence was suppressed to about 5% with HIF-1α siRNA (data not shown). The expression of ODD-Luc protein was accompanied by and correlated with the accumulation of HIF-1α protein under hypoxic treatment (Fig. 2B). Similar results were obtained in the experiments with transient transfection (data not shown). These results suggested that the *5HREp-ODD-luc* gene senses the accumulation of HIF-1α protein and the increase of HIF-1 activity.

We next examined whether the novel reporter gene functions in a tumor xenograft as well as *in vitro*. HeLa/5HRE-ODD-Luc cells were subcutaneously transplanted into the right hind leg of nude mice. The bioluminescence was monitored with an *in vivo* imaging system while blood flow to the xenografts was reduced by ligaturing the right hind leg [15]. A HeLa/EF-Luc tumor xenograft, which constitutively expresses luciferase bioluminescence [15], was prepared in the left hind leg and kept non-ligatured as an internal control for the imaging procedure. The bioluminescence from the HeLa/5HRE-ODD-Luc xenograft increased over time during the ligaturing treatment compared to the non-ligatured internal control (Fig. 2C and D). On the other hand, when the leg with a HeLa/EF-Luc xenograft was ligatured, the bioluminescence was not

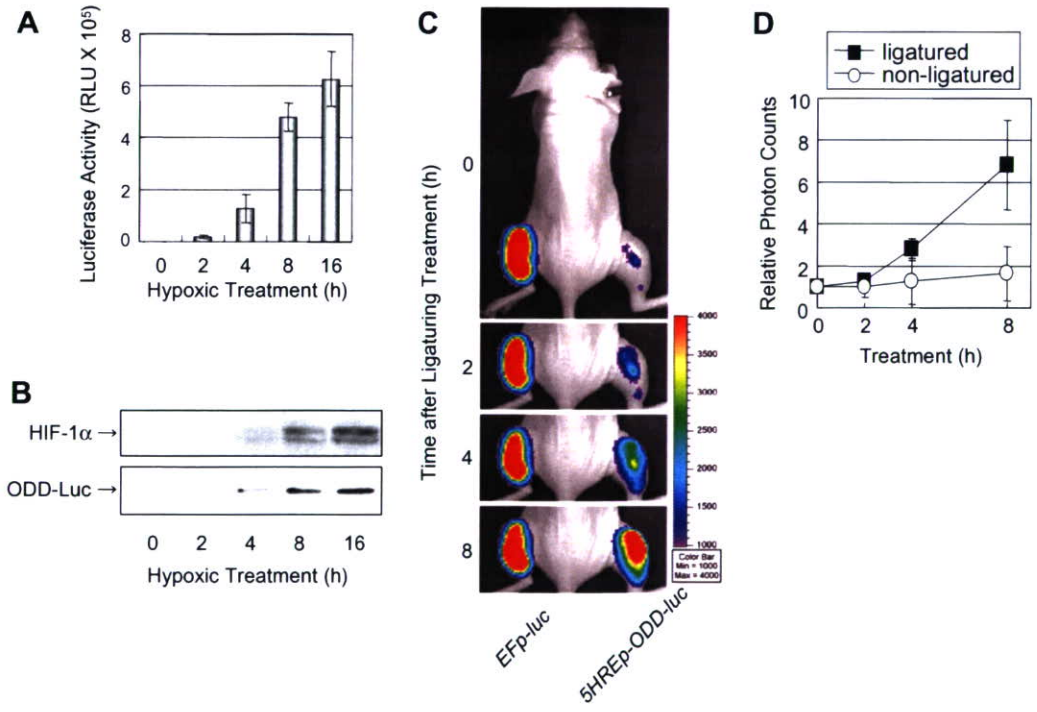

Fig. 2. The *5HREp-ODD-luc* reporter gene reflects increases of HIF-1 activity. (A) HeLa/ 5HRE-ODD-Luc cells were cultured under hypoxic conditions for the period indicated. Bioluminescence was analyzed by luciferase assay. Results are means ± SD, $n = 3$. (B) During and after the hypoxic treatment in (A), expression levels of HIF-1α and ODD-Luc proteins were determined by Western blotting. (C) After blood flow to the HeLa/5HRE-ODD-Luc tumor xenografts in right hind legs was decreased by ligaturing the leg, changes in bioluminescence were sequentially imaged at the indicated time. As an internal control of the imaging procedure, a HeLa/EF-Luc tumor xenograft was prepared in the left hind leg and kept untreated. (D) The bioluminescence (photons/sec/ROI) from ligatured or non-ligatured HeLa/5HRE-ODD-Luc tumor xenografts was quantified. Shown in the graphs are the profiles of the relative photon count after the initial treatment. Results are means ± SD, $n = 5$.

influenced by the treatment (data not shown). All of these results indicate that the accumulation of HIF-1α protein and the resultant increase of HIF-1 activity in tumor xenografts can be monitored as the bioluminescence from the novel reporter gene.

ODD domain suppresses nonspecific bioluminescence from the 5HREp-ODD-luc gene under normoxic conditions

We applied the ODD domain to the novel *5HREp-ODD-luc* reporter gene to circumvent the problems of the conventional *5HREp-luc* gene regarding "normoxic leakage" and "carry over (excess stability of the luciferase protein)". To confirm the effect of added ODD domain, we next examined whether the ODD domain suppresses nonspecific bioluminescence under normoxic conditions. HeLa/5HRE-Luc and HeLa/5HRE-ODD-Luc cells were cultured under normoxic or hypoxic conditions and a luciferase assay and Western blot analysis were conducted. Although the conventional *5HREp-luc* gene showed more than 700-fold increase in hypoxia-responsiveness, it leaked about 1.4×10^3 RLU of bioluminescence without the hypoxic stimuli (Fig. 3A). On the other hand, the novel *5HREp-ODD-luc* gene showed little leakage. Moreover, such a property contributed to an increase in the hypoxia-responsiveness up to about 4.7×10^4 in an all-or-none fashion (Fig. 3A). The Western blot analysis confirmed that the presence of the ODD domain significantly reduced ODD-Luc expression to undetectable level under normoxic conditions (Fig. 3B). These results clearly show that the bioluminescence from the *5HREp-ODD-luc* reporter gene accurately reflects the absolute HIF-1 activity with little background. This conclusion was supported by our finding *in vivo* that the HeLa/5HRE-ODD-Luc tumor xenograft showed less bioluminescence than the HeLa/5HRE-Luc xenograft (Fig. 3C). Taken together, such an expedient property of the ODD domain enabled us to monitor absolute HIF-1 activity in tumor xenografts.

ODD domain enables the 5HREp-ODD-luc gene to sense rapid decreases of HIF-1 activity

We finally examined whether the ODD domain leads to the rapid degradation of the fusion partner, Luc protein, with the identical half-life as HIF-1α protein. After hypoxic treatment, the HeLa/5HRE-ODD-Luc cells were treated with CHX (10 μM) to inhibit protein synthesis thereafter, and cultured under normoxic condition *in vitro*. After such an *in vitro* reoxygenation treatment, the bioluminescence intensity and the expression levels of both HIF-1α protein and luciferase protein were analyzed by luciferase assay and Western blotting, respectively. After the reoxygenation treatments, the ODD-Luc protein level and its bioluminescence were rapidly decreased, and they reflected a rapid degradation of HIF-1α protein (Fig. 4A and B). On the other hand, the luciferase protein and its bioluminescence from the *5HREp-luc* gene remained stable even after the HIF-1α protein was undetectable (Fig. 4A and B). Half-lives of ODD-Luc protein and its bioluminescence were about 10 min after the reoxygenation treatment, while those of luciferase protein were about 12 h (Fig. 4A and B). These results suggest that the bioluminescence from the *5HRE-ODD-luc* gene reflects the rapid disappearance of HIF-1 activity.

To test whether the *5HREp-ODD-luc* gene can sense rapid decreases of HIF-1 activity in tumor xenografts as well as *in vitro*, we performed *in vivo* optical imaging experiments. The blood flow to Hela/5HRE-ODD-Luc tumor xenografts was first reduced by ligaturing the legs and subsequently recovered by releasing the ligature. After such an

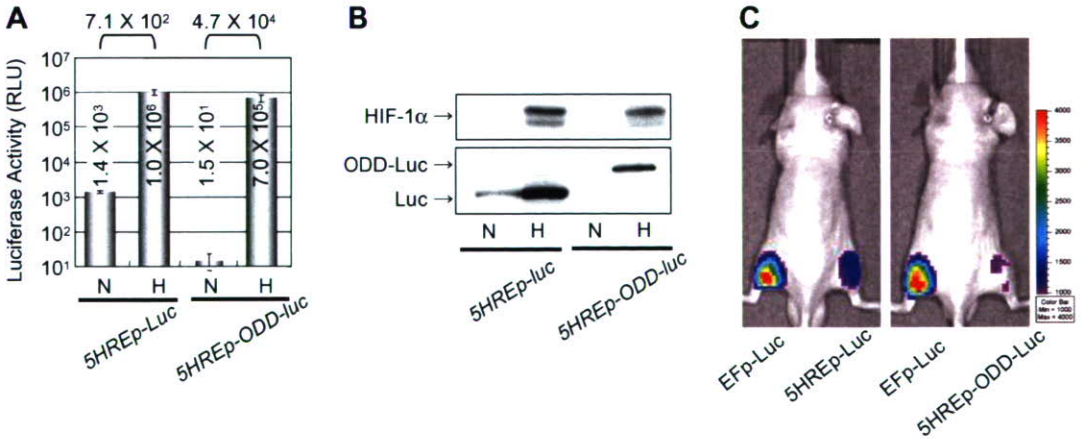

Fig. 3. The ODD domain suppresses nonspecific bioluminescence from the *5HREp-ODD-luc* reporter gene under normoxic conditions. (A) HeLa/5HRE-Luc cells and HeLa/5HREp-ODD-Luc cells were cultured under normoxic or hypoxic conditions for 18 h, and the bioluminescence was analyzed by luciferase assay. Results are means ± SD, $n = 3$. (B) After the same treatments as in (A), expression levels of HIF-1α, luciferase, and ODD-Luc proteins were analyzed by western blotting. (C) Bioluminescence from the HeLa/5HRE-Luc (upper: right hind leg) and HeLa/5HRE-ODD-Luc xenograft was monitored by *in vivo* imaging. HeLa/EF-Luc xenografts were prepared in each left hind leg as an internal control.

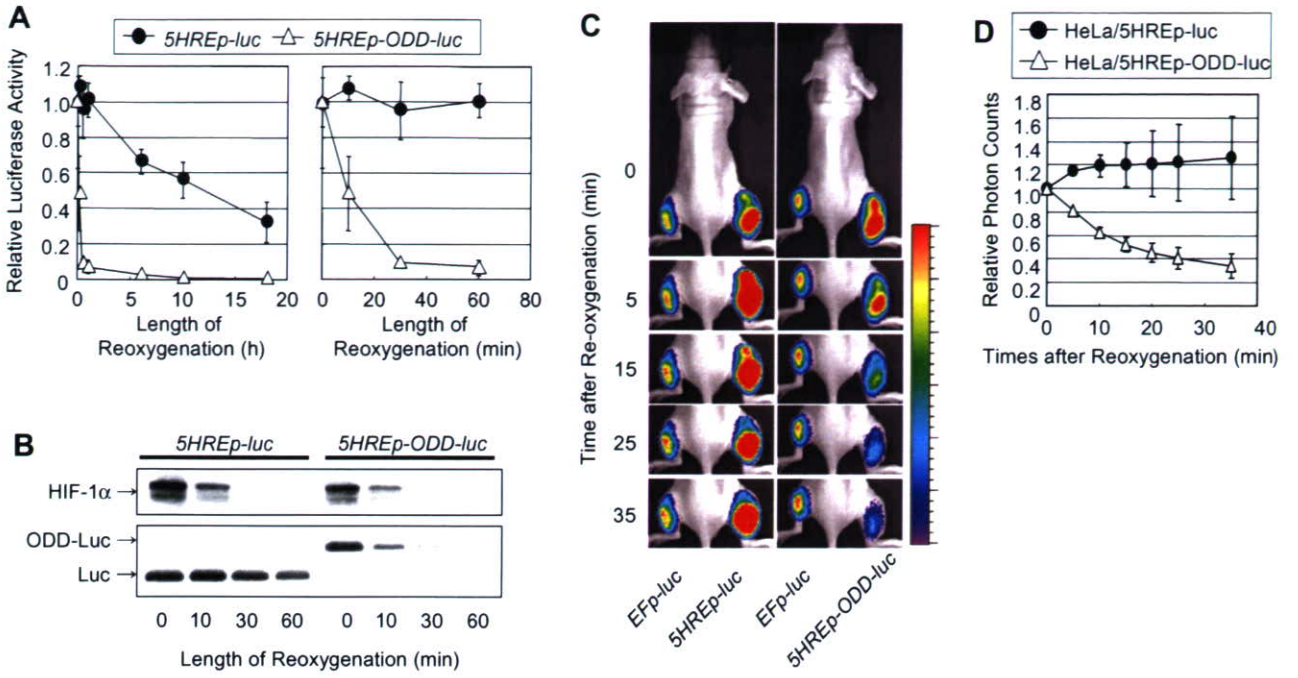

Fig. 4. The ODD domain causes the *5HREp-ODD-luc* gene expression to reflect rapid decreases of HIF-1 activity. (A) After HeLa/5HRE-Luc and HeLa/5HRE-ODD-Luc cells were cultured under hypoxic conditions for 18 h, the cells were treated with CHX (10 μM) to inhibit the protein synthesis thereafter and reoxygenated for the period indicated. Shown in the graphs are the profiles of the relative luciferase activity after the initial reoxygenation treatment. Notably, the relative luciferase activity for the initial 60 min is magnified in the right graph. Results are means ± SD, $n = 3$. (B) HIF-1α, luciferase, and ODD-Luc proteins after the same reoxygenation treatment in (A) were analyzed by Western blotting. (C) The blood flow to HeLa/5HRE-Luc (right hind leg in left mouse) and HeLa/5HRE-ODD-Luc xenografts (right hind leg in right mouse) was reduced by ligaturing the legs for 18 h. The bioluminescence was imaged with an *in vivo* imaging system at the indicated time after the ligature was released ("after reoxygenation"). Luciferin was intravenously injected only once just before the reoxygenation (100 μl of 10 mg/ml sol.). A HeLa/EF-Luc tumor xenograft was transplanted into the left hind leg and kept untreated as an internal control. The bioluminescent intensity of each image was adjusted in order to make the internal control's intensity the same. (D) Changes of the bioluminescent intensity in (C) were quantified. To calculate the relative photon count, the bioluminescence (photons/sec/ROI) from the right tumor was divided by that from the left tumor. Shown in the graphs are the profiles of the relative photon count at each time point after the initial reoxygenation treatment. Results are means ± SD, $n = 5$.

artificial *in vivo* reoxygenation treatment, the bioluminescence was monitored using an *in vivo* imaging system (Fig. 4C). The bioluminescence from the HeLa/5HRE-Luc xenograft was stable during the experimental period. On the other hand, that from the HeLa/5HRE-ODD-Luc xenograft immediately decreased after the reoxygenation treatment. Half-lives of bioluminescence from the HeLa/5HRE-Luc xenograft and the HeLa/5HREp-ODD-Luc xenograft were about 9 h and 17 min after the *in vivo* reoxygenation treatment, respectively (Fig. 4D and data not shown). These results clearly show that the novel *5HREp-ODD-luc* reporter gene senses rapid decreases of HIF-1 activity in tumor xenografts as well as in cultured cells.

Contribution of the present study to future cancer research

In the present study, we constructed a novel *5HREp-ODD-luc* reporter gene and successfully circumvented the problems of "leakage" and "carry-over" in the conventional *5HREp-luc* reporter gene. The fusion of the ODD domain to luciferase gives the same oxygen-dependent instability as HIF-1α protein and led to "little nonspecific bioluminescence under normoxic conditions" and "real-time correlation of the bioluminescence to the increase and decrease of intratumoral HIF-1 activity". Consequently, the novel *5HREp-ODD-luc* reporter gene enabled us to monitor absolute intratumoral HIF-1 activity in real-time. Although we only focused on the development of a novel imaging system for intratumoral HIF-1 activity here, we expect the *5HREp-ODD-luc* gene to facilitate various analyses regarding dynamic change of HIF-1 activity during tumor progression and cancer therapies. Indeed, we recently monitored an unreported dynamism of intratumoral HIF-1 activity during and after radiotherapy (in preparation). Because HIF-1 activity has been well-associated with tumor radioresistance [9,10], the information is worthwhile for the optimization of a protocol for radiotherapy.

In addition, the combination of the 5HRE promoter and ODD domain should have other applications, for example, to hypoxia-targeting gene therapy. Although it has been reported that the 5HRE promoter alone assures a hypoxia-specific effect of adenovirus-mediated cytosine deaminase

gene therapy [17], we expect the combination of 5HRE promoter and ODD domain to further reduce the side-effects in normal tissues. If so, one can increase the dosage of the virus and prodrug, resulting in greater therapeutic benefits without any side-effects. These potential applications are just examples. The *5HREp-ODD-luc* reporter gene is sure to accelerate the pace of cancer research concerning HIF-1, and this knowledge will be very useful to overcome HIF-1-relevant difficulties in cancer therapies.

Acknowledgments

This work was a part of research project by New Energy and Industrial Technology Development Organization (NEDO), Japan, and was supported by a Grant-in-Aid for Cancer Research and for Young Scientist (B) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, and by a Grant-in-Aid for the 2nd and 3rd Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour, and Welfare, Japan.

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British Journal of Cancer (2007) 96, 1871 – 1878

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Adenovirus-mediated hypoxia-targeting cytosine deaminase gene therapy enhances radiotherapy in tumour xenografts

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Hypoxia is closely associated with the radioresistance of tumours; therefore, targeting hypoxic areas is very important for cancer therapy. The aim of this study is to establish such a targeting strategy by applying a bacterial cytosine deaminase (BCD)/5-fluorocytosine (5-FC) gene therapy system and to examine whether the strategy enhances the efficacy of radiotherapy in a tumour xenograft. The hypoxia-responsive promoter 5HREp, in which five copies of the hypoxia-response element (HRE) enhance transcription from a cytomegalovirus minimal promoter, was employed to induce the expression of BCD under hypoxic conditions. The adenoviral vector Ad/5HREp-BCD, encoding the gene *5HREp-BCD*, robustly induced BCD expression under hypoxic conditions and this led to significant cytotoxicity in combination with 5-FC *in vitro*. Intratumoral Ad/5HREp-BCD administration resulted in the expression of BCD at the border between normoxic and necrotic regions. The BCD/5-FC gene therapy enhanced the therapeutic effects of both single (12.5 Gy) and fractionated (3 Gy × 5 days) radiotherapy with few side effects and significantly increased tumour growth doubling time by up to 2.4-fold ($P<0.01$) and 2.5-fold ($P<0.05$), respectively. All of these results suggest that the present BCD/5-FC gene therapy has the ability to specifically target hypoxic tumour cells and significantly improves the control of tumour growth after radiotherapy.
British Journal of Cancer (2007) **96,** 1871 – 1878. doi:10.1038/sj.bjc.6603812 www.bjcancer.com
Published online 22 May 2007

Keywords: tumour hypoxia; hypoxia-response element (HRE); hypoxia-inducible factor-1 (HIF-1); adenovirus; gene therapy; radiotherapy

Translational Therapeutics

The exponential proliferation of cancer cells and the resultant distance that develops between nutritive blood vessels and some tumour cells result in an imbalance in the supply and consumption of oxygen in solid tumours. Such disequilibrium is a major causative factor of tumour hypoxia, a characteristic microenvironment in locally advanced solid tumours (Thomlinson and Gray, 1955; Vaupel *et al*, 1989). The hypoxia is closely associated with malignant phenotypes (Graeber *et al*, 1996), metastasis (Rofstad, 2000), invasion (Pennacchietti *et al*, 2003), and angiogenesis (Harris, 2002). The hypoxic fraction correlates to the resistance to chemotherapy (Teicher, 1994) and radiotherapy (Thomlinson and Gray, 1955; Brown and Wilson, 2004). Therefore, not only has tumour hypoxia been considered an adverse prognostic indicator, but also, a hypoxia-targeting strategy is becoming increasingly important to overcome these problems (Teicher, 1994; Harris, 2002; Brown and Wilson, 2004).

Under hypoxic conditions, hypoxia-inducible factor-1 (HIF-1) plays a pivotal role in inducing the expression of various genes (Semenza, 2001). Hypoxia-inducible factor-1 is a heterodimeric transcription factor composed of an α-subunit (HIF-1α) and a β-subunit (HIF-1β) (Wang *et al*, 1995). The expression of HIF-1α is regulated in an oxygen-dependent manner mainly at the post-translational level and is responsible for the regulation of HIF-1's activity (Kallio *et al*, 1997). Proline residues in the oxygen-dependent degradation domain of HIF-1α protein are hydroxylated under normoxic conditions (Jaakkola *et al*, 2001). The modified HIF-1α protein is ubiquitinated by E3 ubiquitin-protein ligases containing the von Hippel-Lindau tumour suppressor protein (pVHL) and rapidly degraded by the 26S proteasome (Jaakkola *et al*, 2001). On the other hand, the rate at which proline was hydroxylated decreased under hypoxic conditions, resulting in a reduced rate of ubiquitination and subsequent degradation (Jaakkola *et al*, 2001). The stabilised HIF-1α interacts with the constitutively expressed HIF-1β protein and induces the gene expression of erythropoietin (Wang and Semenza, 1993), VEGF (Forsythe *et al*, 1996), and others (Semenza, 2001). The induction is triggered by the interaction of HIF-1 with its cognate DNA recognition site, the hypoxia-response element (HRE) (Norris and Millhorn, 1995; Forsythe *et al*, 1996). An increased level of HIF-1α in the tumour and the resultant upregulation of HIF-1 activity as well as tumour hypoxia have been associated with tumour malignancy, aggressive tumour growth, tumour radioresistance and a poor prognosis (Powis and Kirkpatrick, 2004).

Extensive efforts have focused on the development of biological approaches to deal with tumour hypoxia (Semenza, 2003; Brown and Wilson, 2004). One of the most striking advances is the development of artificial hypoxia-responsive promoters (Greco

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All of our *in vivo* animal experiments were approved by the Animal Research Committee of Kyoto University, and the procedures were consistent with the United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) Guidelines for the welfare of animals in experimental neoplasia (Second Edition).
Revised 16 April 2007; accepted 24 April 2007; published online 22 May 2007

et al, 2000), in which the HRE(s) has been utilised as a transcriptional enhancer. Many groups have reported that a tandem repeat of HREs enhances gene expression under hypoxic conditions (Greco *et al*, 2000). Above all, the 5HRE promoter (5HREp), in which five copies of the HRE enhance transcription from a cytomegalovirus (CMV) minimal promoter, enhances gene expression more than 500-fold under hypoxic conditions *in vitro* (Shibata *et al*, 1998, 2000; Greco *et al*, 2000). Optical imaging of tumour hypoxia by using the 5HREp-luciferase gene and the *5HREp*-green fluorescent protein (GFP) gene has proved the potential of the promoter *in vivo* as well as *in vitro* (Vordermark *et al*, 2001; Harada *et al*, 2005; Liu *et al*, 2005). Hypoxia-specific targeting was also accomplished *in vivo*, when cytotoxic genes or therapeutic genes, such as for apoptotic factors or prodrug-activating enzymes, were inserted downstream of the hypoxia-responsive promoters (Greco *et al*, 2000; Koshikawa *et al*, 2000; Patterson *et al*, 2002; Shibata *et al*, 2002; Binley *et al*, 2003; Ogura *et al*, 2005). However, all of these *in vivo* experiments were conducted using stable transfectants with each hypoxia-responsive gene. In other words, no one has examined whether 5HREp would function in a *trans*-acting gene therapy strategy.

In the present study, we utilised 5HREp (Shibata *et al*, 2000) and a prodrug-activating gene, bacterial cytosine deaminase (BCD) (Mullen *et al*, 1992; Miller *et al*, 2002), and successfully established an adenovirus-mediated gene therapy strategy for tumour hypoxia. We used this strategy to determine whether the specific targeting of tumour hypoxia by gene therapy improves the efficacy of radiotherapy in a tumour xenograft.

Translational Therapeutics

MATERIALS AND METHODS

Cell culture

The human cervical epithelial adenocarcinoma cell line HeLa and the human pancreatic carcinoma cell line MIA PaCa-2 were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The human pancreatic carcinoma cell line CFPAC-1 was maintained in Iscove's modified Dulbecco's medium (IMDM) with 10% fetal bovine serum. The human colon carcinoma cell lines WiDr and HT29 were maintained in RPMI-1640 medium with 10% fetal bovine serum. All cell lines were purchased from American Type Culture Collection. For normoxic incubation, the cells were incubated in a well-humidified incubator with 5% CO_2 and 95% air at 37°C.

Plasmid DNA

To construct the plasmid pEF/BCD, which constitutively expresses a BCD protein fused to a myc epitope tag under the control of the EF-1α promoter, a DNA fragment for the *Escherichia coli codA* gene, which encodes the enzyme cytosine deaminase, was amplified by PCR and inserted between *NcoI* and *NotI* recognition sites of the vector pEF/myc/cyto (Invitrogen, Carlsbad, CA, USA). To construct the plasmid p5HRE/BCD, which induces the expression of the BCD-myc fusion protein under hypoxic conditions, a DNA fragment for 5HREp was obtained by digestion with *KpnI* and *NcoI* from the vector, 5HRE/hCMVmp (Shibata *et al*, 2000), inserted between *KpnI* and *NcoI* recognition sites of pEF/BCD, and substituted for the constitutive EF-1α promoter.

Stable transfectants

To establish stable transfectants, HeLa/EFp-BCD and HeLa/5HREp-BCD, HeLa cells (1×10^5) were transfected with pEF/BCD and p5HRE/BCD, respectively, by a modified calcium-phosphate method (Chen and Okayama, 1987, 1988). Twenty-four hours after the transfection, the culture medium was refreshed with selection medium containing 5 $\mu g\,ml^{-1}$ of blasticidine for HeLa/EFp-BCD cells or 400 $\mu g\,ml^{-1}$ of G418 for HeLa/5HREp-BCD cells. Each antibiotic-resistant cell culture was directly used for both the Western blot analysis and the *in vitro* cell proliferation assay.

Construction, amplification, and infection of the adenovirus

To construct cosmid vectors, pAxcw/EFp-BCD and pAxcw/5HREp-BCD, DNA fragments for *EFp-BCD* and *5HREp-BCD* were prepared from pEF/BCD and p5HRE/BCD, respectively, by digestion with *KpnI* and *BamHI*, blunted and inserted into the *SwaI* recognition site of the cosmid vector pAxcw (TaKaRa, Tokyo, Japan). The recombinant adenoviruses, Ad/EFp-BCD and Ad/5HREp-BCD, were generated by COS-TPC methods (Miyake *et al*, 1996) using an adenovirus expression kit according to the manufacturer's instructions (TaKaRa). For large-scale preparations, the adenoviruses were amplified in a transformed human embryonic kidney cell line, 293, and purified by two steps of caesium chloride density centrifugation. Viral titers were measured in a limiting-dilution bioassay using 293 cells. Cells (1×10^5 cells per dish) were seeded onto a 60 mm dish and treated with Ad/EFp-BCD or Ad/5HREp-BCD for 1 h. Then, the adenovirus-containing medium was replaced with one without the virus.

Western blot analysis

The stable transfectants and the virus-infected cells were seeded in 60 mm glass dishes (2×10^5 cells per dish), put into pre-warmed aluminium chambers, and flushed with hypoxic gas (95% N_2, 5% CO_2) for 30 min. Then, tightly sealed chambers were incubated at 37°C for 16 h for the hypoxic treatment. The cells were harvested in RIPA lysis buffer (10% SDS, 2 M Tris-HCl, pH 7.5, and 1% Triton X) supplemented with a protease inhibitor, Mini complete (Roche, Basel, Switzerland). The lysates were sheared using a syringe with a 23-gauge needle, and the protein concentration was determined using the DC Protein assay kit (Bio-Rad). Twenty micrograms of total protein was electrophoresed on a 12% SDS polyacrylamide gel, transferred onto PVDF membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and blocked with 5% non-fat milk in Tris-buffered saline. The BCD protein fused to the myc epitope tag was detected with monoclonal anti myc-tag antibody (Cell Signaling Technology Inc., Danvers, MA, USA) and anti mouse IgG horseradish peroxidase-linked whole antibody (GE Healthcare Bio-Sciences Corp.) using an ECL-PLUS system (GE Healthcare Bio-Sciences Corp.) according to the manufacturer's instructions.

In vitro cell proliferation assay

The stable transfectants and the virus-infected cells were seeded in triplicate into 96-well plates (1×10^3 cells per well) and incubated with various concentrations of 5-fluorocytosine (5-FC) (Sigma Chemical Co., St Louis, MO, USA) for 24 h under normoxic or hypoxic conditions. For the hypoxic treatment (<0.02% of oxygen), the cells were treated in a hypoxic chamber, BACTRON-II (Sheldon Manufacturing Inc., Cornelius, OR, USA). The cells were additionally incubated under normoxic conditions for 24 h. The culture medium was then changed to one without 5-FC, and the cells were cultured for 48 h under the normoxic conditions. Cell growth inhibition was quantified by colorimetric assay using a CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Tumour-bearing mice

A suspension of HeLa cells (2×10^6 cells/100 μl of PBS) was subcutaneously inoculated into the right hind leg of a 6-week-old nu/nu BALB/c mice (Charles River, Tokyo, Japan).

Immunohistochemical analysis

The adenovirus Ad/EFp-BCD or Ad/5HREp-BCD was intratumorally injected into the HeLa tumour xenografts, when the xenografts developed to approximately 150–200 mm^3. Four days later, pimonidazole hydrochloride (Natural Pharmacia International Inc., Belmont, MA, USA) was intraperitoneally (i.p.) injected into the tumour-bearing mice (60 mg kg^{-1}). Ninety minutes later, the tumours were surgically excised, immediately fixed in 10% formalin neutral buffer solution (pH = 7.4; Wako Pure Chemical Industries Inc., Osaka, Japan), and embedded in paraffin. To detect pimonidazole and BCD-myc, paraffin-embedded sections were treated with anti-pimonidazole (Natural Pharmacia International Inc.) and anti-*c*-myc antibody (Santa Cruz, CA, USA), respectively, and stained using an indirect immunoperoxidase detection method (DakoCytomation, Carpinteria, CA, USA), according to the manufacturer's instructions. Counterstaining with haematoxylin was also carried out. Paraffin-embedded serial sections were also stained with haematoxylin–eosin (HE).

Radiation conditions

The tumour-bearing mice were irradiated at 1.468 Gy min^{-1} with an X-ray irradiation machine (SHIMADZU, Kyoto, Japan). All the tumour-bearing mice were restrained and shielded with a specially designed lead apparatus that allowed local irradiation to the tumour on the right hind leg.

Growth delay assays

When the tumour xenografts developed to approximately 150–200 mm^3, the tumour-bearing mice were randomly divided into five treatment groups: (1) a sham-treated group, (2) an Ad & 5-FC group, (3) an ionising radiation (IR) group, (4) an IR & 5-FC group and (5) an Ad & 5-FC & IR group. In the single irradiation experiment, 2×10^9 PFU of adenovirus was intratumorally injected into the mice of the Ad & 5-FC and Ad & 5-FC & IR groups on day 0. 5-FC (500 mg kg^{-1}) was i.p. injected into the mice of the Ad & 5-FC, IR & 5-FC, and Ad & 5-FC & IR groups on both day 1 and day 2. Irradiation (12.5 Gy) was applied to the mice of the IR, IR & 5-FC, and Ad & 5-FC & IR groups 12 h after the injection of 5-FC on day 1. In the fractionated irradiation experiment, the adenovirus was intratumorally injected into the mice of the Ad & 5-FC and Ad & 5-FC & IR groups on day 0. 5-Fluorocytosine was administered daily from day 1 to day 5 to the mice of the Ad & 5-FC, IR & 5-FC, and Ad & 5-FC & IR groups. Irradiation was applied 12 h after the injection of 5-FC daily from day 1 to day 5 to the mice of the IR, IR & 5-FC, and Ad & 5-FC & IR groups (3 Gy × 5 days). For the negative control, PBS was injected instead of the adenovirus and the 5-FC. The tumour size was measured with calipers, and the tumour volume was calculated as $0.5LW^2$.

Statistical analysis

The statistical significance of differences was determined using the Student's *t*-test ($P < 0.05$).

RESULTS

Establishment of a hypoxia-dependent prodrug-activating system

To establish a hypoxia-targeting gene therapy strategy, we first constructed a plasmid, p5HRE/BCD, encoding the *5HREp-BCD* gene (Figure 1A). Shibata *et al* (2000) employed 5HREp to induce the therapeutic gene expression specifically under hypoxic conditions. The *BCD* gene was used as the prodrug-activating gene, because the intratumoral production of 5-fluorouracil by the BCD/5-FC system is effective for cancer therapy (Mullen *et al*, 1992; Miller *et al*, 2002). HeLa cells were stably transfected with p5HRE/BCD and the hypoxia dependency of the BCD expression was examined by Western blot analysis (Figure 1B). The stable transfectant, HeLa/5HREp-BCD, expressed the BCD protein only under hypoxic conditions, while the HeLa/EFp-BCD cells, which had been expected to express constitutively the protein, indeed expressed BCD regardless of the conditions. We next examined whether the hypoxia-dependent BCD expression led to the hypoxia-specific cytotoxicity. The HeLa/5HREp-BCD cells were treated with various concentrations of 5-FC under normoxic or hypoxic conditions, and the growth inhibitory effects were assessed by MTS assay. Significant growth inhibition was observed only under hypoxic conditions ($P < 0.05$ with 0.1 mg ml^{-1} of 5-FC, $P < 0.01$ with 1 and 10 mg ml^{-1} of 5FC). There was no significant inhibition observed under normoxic conditions (Figure 1C). On the other hand, HeLa/EFp-BCD cells showed hypoxia-independent sensitivity to the 5-FC treatment (Figure 1D). Thus, we confirmed that the 5HREp-dependent BCD/5-FC strategy led to the hypoxia-specific cytotoxicity.

Ad/5HREp-BCD-mediated cytotoxicity under hypoxic conditions

We decided to use an adenovirus to transduce the *5HREp-BCD* gene into tumour cells *in vivo*, because the adenovirus is one of the most effective vectors with which to accomplish *trans*-gene expression. We constructed a cosmid vector, pAxcw/5HREp-BCD (Figure 2A), and obtained the adenovirus, Ad/5HREp-BCD, by the

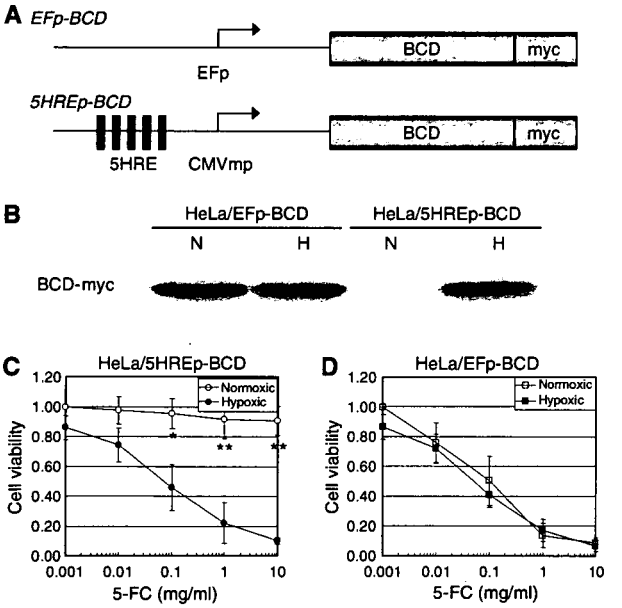

Figure 1 Hypoxia-responsive BCD expression and 5-FC sensitivity. (**A**) Schematic diagrams of the *EFp-BCD* gene constitutively expressing the BCD (top) and the *5HREp-BCD* gene hypoxia-dependently expressing the BCD (bottom). The BCD coding gene was fused to the myc epitope in frame. (**B**) Western blot analysis of BCD-myc expression in HeLa/EFp-BCD cells or HeLa/5HREp-BCD cells under normoxic (N) or hypoxic (H) conditions. (**C** and **D**) Cell proliferation assay of HeLa/5HREp-BCD cells (**C**) and HeLa/EFp-BCD cells (**D**). The cells were treated with various concentrations of 5-FC under normoxic (open) or hypoxic (solid) conditions. Cell viability was calculated as the ratio of the absorbance value in each of the conditions against that in medium with 0.001 mg ml^{-1} of 5-FC under normoxic conditions. Results are the mean ± s.d. ($n = 3$). *$P < 0.05$. **$P < 0.01$.

COS-TPC methods (Miyake *et al*, 1996). To examine whether Ad/5HREp-BCD showed hypoxia-dependent BCD expression, we performed a Western blot analysis (Figure 2B). HeLa cells were infected with Ad/5HREp-BCD at a MOI of 10-100 and cultured under normoxic or hypoxic conditions. The BCD protein was expressed only under hypoxic conditions. The amount of BCD protein expressed and the ratio of the expression under hypoxia to that under normoxia increased with the increase in the MOI. On the other hand, HeLa cells infected with Ad/EFp-BCD constitutively expressed BCD protein regardless of oxygen conditions (Figure 2B).

We next evaluated the hypoxia dependency and the therapeutic efficacy of the Ad/5HREp-BCD-mediated strategy *in vitro*. The virus-infected HeLa cells were exposed to various concentrations of 5-FC under normoxic or hypoxic conditions, and the growth inhibitory effect was examined by MTS assay (Figure 3A). The cell proliferation was significantly inhibited under hypoxic conditions compared to normoxic conditions when the cells were treated with Ad/5HREp-BCD (MOI = 100) and the higher concentration of 5-FC. Likewise, MIA PaCa-2 and WiDr cells showed hypoxia-dependent sensitivity to the adenovirus-mediated BCD/5-FC treatment (Figure 3B and C). On the other hand, proliferation was inhibited under both normoxic and hypoxic conditions, when the cells were infected with Ad/EFp-BCD (MOI = 100). All of the *in vitro* experiments clearly indicate that our system functioned as we desired.

Hypoxia-specific BCD expression after intratumoral Ad/5HREp-BCD injection

We examined whether Ad/5HREp-BCD induces the expression of BCD in hypoxic regions of the tumour xenograft. The virus (1×10^9 pfu) was intratumorally injected into HeLa tumour xenografts, and the regions expressing BCD were compared to those stained with a marker of hypoxia, pimonidazole (Durand and Raleigh, 1998). The immunohistochemical analysis showed that the hypoxic cells stained with pimonidazole were located about 100 μm from a tumour blood vessel, and a robust expression of BCD was also observed there (Figure 4A-C). On the other hand, remarkable BCD expression was observed in well-oxygenated viable regions after intratumoral injection of Ad/EFp-BCD (Figure 4D and E). These results suggest that the *trans*-gene expression of BCD in hypoxic tumour cells can be achieved by the intratumoral administration of the adenovirus Ad/5HREp-BCD.

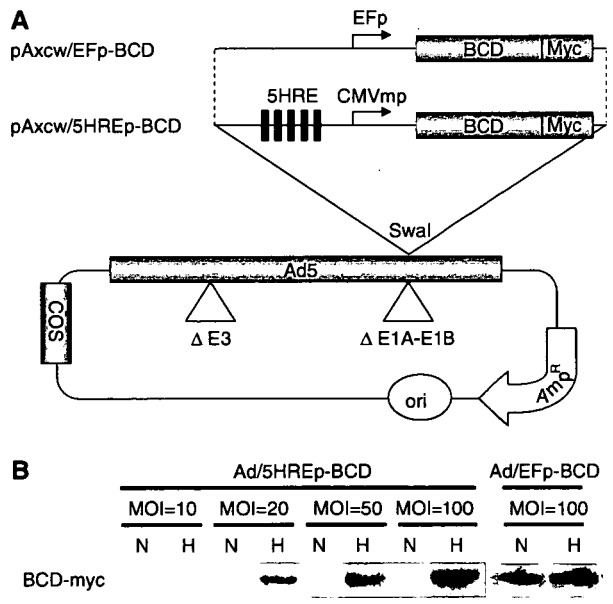

Figure 2 Adenovirus-mediated BCD expression under hypoxic conditions. (**A**) Schematic diagrams of the cosmid vectors, pAxcw/EFp-BCD (top) and pAxcw/5HREp-BCD (bottom), encoding *EFp-BCD* and *5HREp-BCD*, respectively. Ori = replication origin; Amp^R = ampicillin-resistance gene; COS = cos (phage λ sequences) region (**B**) Western blot analysis of BCD-myc expression by using anti myc-tag antibody. HeLa cells were infected with Ad/EFp-BCD or Ad/5HREp-BCD at the indicated MOI, and exposed to normoxic (N) or hypoxic (H) conditions.

Improvement of radiotherapy by Ad/5HREp-BCD-mediated gene therapy

The *in vitro* cell proliferation assay (Figure 3) and the immunohistochemical analysis (Figure 4) led us to expect a hypoxia-specific therapeutic effect of the Ad/5HREp-BCD, and 5-FC gene therapy. Actually, we confirmed an advantage of 5HREp concerning side effects on normal tissues. The Ad/5HREp-BCD/5-FC gene therapy caused no obvious side effects, while the Ad/EFp-BCD/5-FC gene therapy, despite the local administration, caused significant weight loss (Figure 5A) and severe diarrhea (data not shown). This result indicates that our system functioned, as we desired.

We next examined whether the combination of the Ad/5HREp-BCD/5-FC gene therapy with radiotherapy produced a synergistic antitumour effect. We treated HeLa tumour xenografts with the gene therapy (Ad & 5-FC) and/or radiotherapy (IR) and carried out growth delay assays (Figure 5B). We intentionally chose a relatively low dose of Ad & 5-FC, which had minimal effects on the tumour growth rate. Therefore, tumour growth after the gene therapy alone (Ad & 5-FC group) was not significantly suppressed compared to that after sham-treatment (sham-treated group). On the other hand, combined with IR (Ad & 5-FC & IR group), the gene therapy strikingly suppressed tumour growth as compared to radiotherapy alone (IR group). The period taken for tumour growth to increase two-fold from the initial volume (tumour growth doubling time, TGDT) more clearly shows the therapeutic effect of the treatment (Table 1). The TGDT after gene therapy alone (Ad & 5-FC group) was 13.2 ± 5.6 days, which is not significantly longer than that after sham-treatment (8.2 ± 3.1 days; $P = 0.144$). On the other hand, the combination of gene therapy with radiotherapy (Ad & 5-FC & IR) prolonged the TGDT to 47.2 ± 16.8 days, which was about 2.4-fold longer than that after radiotherapy alone (IR group; 19.4 ± 4.8 days; $P < 0.01$). Thus, we confirmed that the adenovirus-mediated and hypoxia-targeting gene therapy significantly enhances the effect of radiotherapy.

Similar results were obtained in the experiment using the fractionated irradiation (3 Gy × 5 fractions: Figure 5C). The TGDT after gene therapy alone (Ad & 5-FC group) was 13.0 ± 4.4 days, which is not significantly longer than that after sham treatment (9.8 ± 5.8 days; $P = 0.148$). On the other hand, the TGDT after the fractionated radiotherapy (IR) was 17.0 ± 3.7 days, which was significantly delayed by the combination with the gene therapy (Ad & 5-FC & IR group) to 43.3 ± 23.8 days (Table 1; $P < 0.05$). These results further strengthen the conclusion that hypoxia targeting by gene therapy improves the efficacy of radiotherapy.

DISCUSSION

In the present study, we established a hypoxia-targeting strategy by applying a BCD/5-FC gene therapy system and examined whether the strategy enhances the efficacy of radiotherapy in a tumour xenograft.

Because tumour hypoxia has been recognised as a tumour-specific microenvironment, recent research has tried to exploit it as a crucial target for cancer therapy (Harris, 2002; Semenza, 2003;

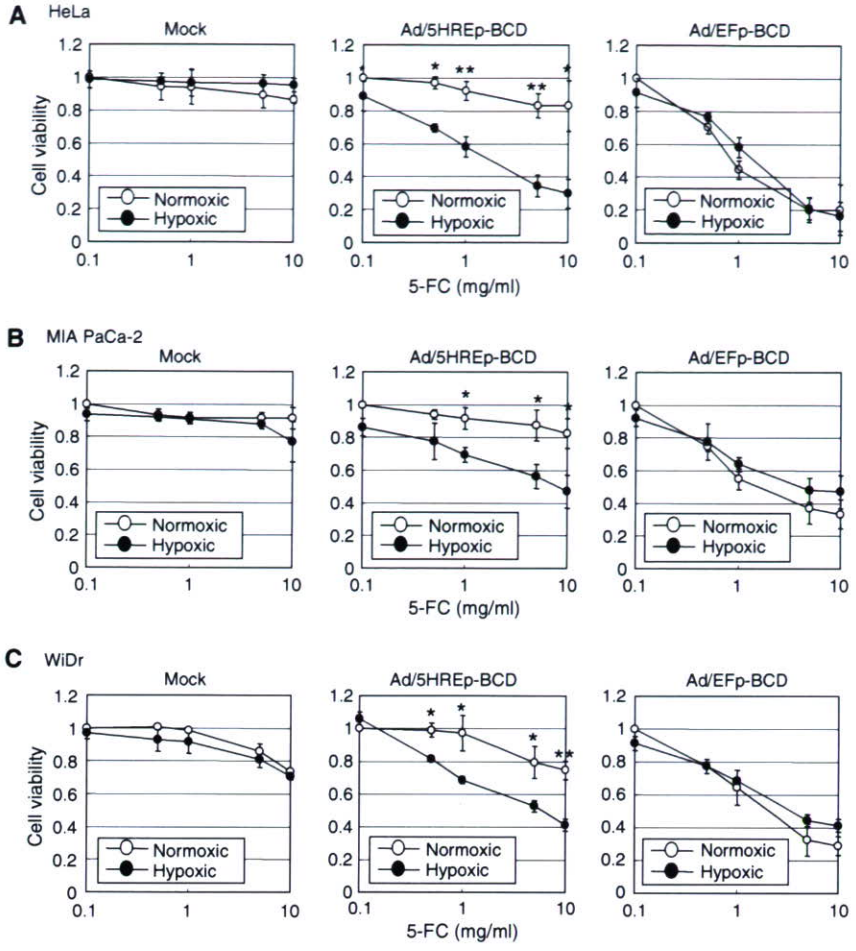

Figure 3 Ad/5HREp-BCD-mediated cytotoxicity. (**A**) HeLa, (**B**) MIA PaCa-2, and (**C**) WiDr cells were infected with the adenovirus, Ad/EFp-BCD or Ad/5HREp-BCD, and treated with various concentrations of 5-FC under normoxic (open) or hypoxic (solid) conditions. Cell viability was calculated as the ratio of the absorbance value under each of the conditions against that in medium with 0.1 mg ml^{-1} of 5-FC under normoxic conditions. The same experiment was conducted with mock infection. Results are the mean ± s.d. ($n = 3$). *$P < 0.05$. **$P < 0.01$.

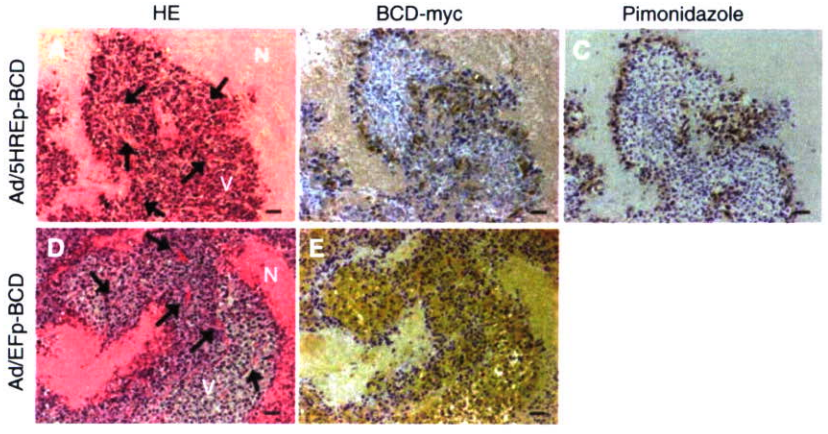

Figure 4 Immunohistochemical analysis of BCD expression in virus-injected tumour xenografts. The tumour xenograft of HeLa cells was intratumorally injected with Ad/5HREp-BCD (**A–C**) or Ad/EFp-BCD (**D** and **E**). Serial sections of the xenograft were subjected to HE staining (**A** and **D**), and to immunohistochemical analysis with anti-c-myc antibody for the detection of BCD-myc (**B** and **E**), and with anti-pimonidazole antibody (**C**). Bar = 100 μm. N = necrotic tumour tissue; V = well-oxygenated viable tumour tissue; arrow = blood vessel.

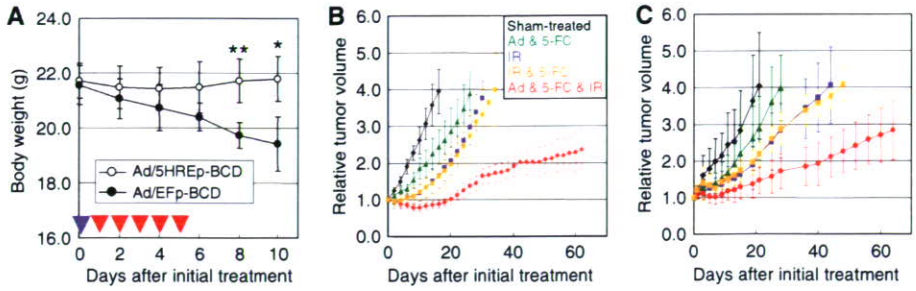

Figure 5 Synergistic antitumour effect of a combination of gene therapy with IR treatment. (**A**) Ad/EFp-BCD or Ad/5HREp-BCD was intratumorally injected into tumor-bearing mice on day 0 (blue arrow head), and 5-FC was administered daily from day 1 to day 5 (red arrow head). Body weight of the tumour-bearing mice was measured during and after the treatment. The results are the mean of six independent mice ± s.d. $*P<0.05$. $**P<0.01$. (**B** and **C**) Tumour-bearing mice in the Ad & 5-FC group and Ad & 5-FC & IR group were administered Ad/5HREp-BCD. Those in the Ad & 5-FC group, IR & 5-FC group, and Ad & 5-FC & IR group were administered 5-FC. The tumour xenografts in the IR group, IR & 5-FC group, and Ad & 5-FC & IR group were locally exposed to irradiation with a single dose of 12.5 Gy (**B**) or daily dose of 3 Gy for 5 days (**C**). (See Materials and methods for details.) Tumour volume was measured with calipers and calculated as $0.5\ L\ W^2$. Relative tumour volume indicates the ratio of the tumour volume on each day to the corresponding volume on day 0. The results are the mean of six independent tumours ± s.d.

Table 1 Statistical analysis of TGDT

	Single (12.5 Gy)	Fractionated (3 Gy × 5)
Sham-treatment	8.2 ± 3.1	9.8 ± 5.8
Ad & 5-FC	13.2 ± 5.6 ($P=0.144$ *vs* Sham)	13.0 ± 4.4 ($P=0.148$ *vs* Sham)
IR	19.4 ± 4.8	17.0 ± 3.7
IR & 5-FC	19.8 ± 3.4 ($P=0.865$ *vs* IR)	16.8 ± 2.5 ($P=0.644$ *vs* IR)
Ad & 5-FC & IR	47.2 ± 16.8 ($P<0.01$ *vs* IR)	43.3 ± 23.8 ($P<0.05$ *vs* IR)

Abbreviations: 5-FC = 5-fluorocytosine; TGDT = tumour growth doubling time. TGDT was calculated as the mean of the days, on which relative tumor volume of each tumor reached 2-fold of that on day 0. Data were based on the results of the growth delay assays in Figures 5B and C with single (12.5 Gy) and fractionated (3 Gy × 5) irradiation, respectively. Results are the mean of the days ± s.d. ($n=6$).

Brown and Wilson, 2004). In this regard, the hypoxia-specific gene therapy strategy has been focused on, and artificial hypoxia-responsive promoters have been developed using various kinds of HREs, such as murine PGK-1 HRE, human erythropoietin HRE, and human VEGF HRE (Greco *et al*, 2000). Above all, 5HREp showed the best hypoxia-responsiveness and exhibited a more than 500-fold increase in luciferase activity in response to hypoxic stimuli (Shibata *et al*, 2000). The absolute level of luciferase activity from 5HREp under the hypoxic conditions reached the same level as that from the CMV-driven promoter under normoxic conditions (Shibata *et al*, 2000). Consistent with these previous reports, the expression of BCD was robustly induced under hypoxic conditions in our plasmid based and adenovirus-based Western blot analysis. This induction actually led to significant hypoxia-dependent cytotoxicity in our cell proliferation assay.

The sensitivity of each cell line to the Ad/5HREp-BCD/5-FC treatment varied in the present cell proliferation assay (Figure 3; compare the viability of each cell line at MOI = 100). Among the cell lines tested, HeLa cells exhibited the highest hypoxia dependency concerning sensitivity to the treatment. On the other hand, a human colon carcinoma cell line, HT29, and a human pancreatic carcinoma cell line, CFPAC-1, showed little therapeutic efficacy (Supplementary Figure S1A and B). We hypothesised that this variability might be caused by the difference in the efficiency of adenoviral infection in each cell line, because it was reported that cells showed different infection efficiencies and CFPAC-1 cells had the lowest transduction efficiency among cells tested (Bouvet *et al*, 1998). We performed a chemiluminescent β-gal assay to analyse the efficiency of the adenoviral infection and confirmed that HeLa cells showed the highest, and HT29 and CFPAC-1 cells, a much lower, infection efficiency (Supplementary Table S1). Moreover, when we transfected HT29 and CFPAC-1 cells with p5HRE/DsRed2 plasmid (not an adenovirus), we confirmed the presence of hypoxia-dependent red fluorescence, indicating that the 5HREp works in these cells (Supplementary Figure S2). Therefore, we concluded that the low infection efficacy of the adenovirus was responsible for the weak therapeutic efficacy in HT29 and CFPAC-1 cells. These results indicate that, although hypoxia is a common feature of solid tumours, our hypoxia-targeting system cannot target all tumour hypoxia without an excellent vector.

To measure the damage to normal tissue after hypoxia-targeting treatment, Binley *et al* (2003) applied a hypoxia-responsive thymidine kinase/ganciclovir (TK/GCV) strategy and evaluated the activity of lactate dehydrogenase (LDH) as an indicator of liver dysfunction. Hypoxia-dependent TK expression and GCV treatment caused no irregularity in LDH levels. On the other hand, constitutive TK expression from a CMV promoter and GCV treatment significantly elevated LDH levels in mice. These results suggest that a hypoxia-responsive promoter would facilitate target-specificity and so reduce the side effects on well-oxygenated normal tissues. Consistent with these reports, we did not observe any obvious side effects after the Ad/5HREp-BCD/5-FC gene therapy. On the other hand, after the Ad/EFp-BCD/5-FC treatment, we observed significant weight loss and severe diarrhea, despite the local administration (Figure 5A). These results strengthen further the argument that tumour hypoxia is a specific therapeutic target and our 5HRE system has the advantage of specifically targeting it.

To determine whether the specific targeting of tumour hypoxia by the gene therapy strategy improves the efficacy of radiotherapy in a tumour xenograft, we performed growth delay assays. The gene therapy synergistically kept tumour growth suppressed in combination with the single (12.5 Gy) and the fractionated (3 Gy × 5 fractions) radiotherapy. These results were consistent with the report that a combination of HRE-driven P450R expression and tirapazamine significantly increased the efficacy of radiotherapy *in vivo* (Cowen *et al*, 2004). The data together with ours definitely support that hypoxia-targeting gene therapy combined with radiotherapy is a promising approach to cancer treatment.

Although BCD expression from the *5HREp-BCD* gene was not observed under normoxic conditions in the present Western blotting (Figures 1B and 2B), the cells showed slight but clear sensitivity to a high concentration of 5-FC even under normoxic

conditions (Figure 3). This sensitivity was observed regardless of infection with the adenovirus *in vitro* (Figure 3; compare MOI = 0–100 in each cell), indicating that an excess dose of 5-FC itself results in BCD-independent cytotoxicity. In our growth delay assays, a significant difference was not observed in TGDT between the IR group and the IR & 5-FC group (without adenovirus administration) (Figure 5B and C and Table 1), indicating that the dose of 5-FC was not excessive, or rather was moderate in our *in vivo* studies. In such an experimental setting, tumour growth in the Ad & 5-FC & IR group was significantly delayed compared to that in the IR group (Figure 5B and C and Table 1). All of these results strongly suggest that the synergistic therapeutic effect of Ad & 5-FC & IR treatment was dependent on the expression of BCD and was caused by the conversion of 5-FC to cytotoxic 5-FU.

Hypoxia-inducible factor-1 plays important roles in regulating tumour radiosensitivity, and therefore, it has been recognised as a potentially promising target for tumour radiosensitisation (Moeller *et al*, 2004; Moeller and Dewhirst, 2006). Because transcription from 5HREp mainly depends on HIF-1 activity, BCD expression from Ad/5HREp-BCD should be induced in the cells with increased HIF-1 activity. In this regard, the gene therapy should have targeted the tumour cells with increased HIF-1 activity and enhanced the therapeutic effect of radiotherapy.

We previously used 5HREp to image hypoxic cells in tumour xenografts (Harada *et al*, 2005; Liu *et al*, 2005). These studies were conducted using tumour xenografts, in which a hypoxia-responsive gene, such as the *5HREp-luciferase* or the *5HREp-GFP* gene, had been previously set, but never using vectors responsible for the *trans*-gene delivery. In the present immunohistochemical analysis, we confirmed the intratumoral expression of BCD after the direct administration of the adenoviral vector into tumour xenografts. The expression was limited to and near the pimonidazole-positive hypoxic regions. Moreover, the BCD was biologically active and indeed led to the antitumour effect we desired. These results represent great progress toward the clinical use of this hypoxia-targeting strategy. However, the most important problem still remains; after the systemic intravenous administration of Ad/5HREp-BCD, we did not detect the expression of BCD in the tumour xenografts in the immunohistochemical analysis (data not shown). For the clinical application of the present gene therapy strategy, the development of a novel gene delivery technology is the next issue to be addressed, although work on this has met with minimal success.

ACKNOWLEDGEMENTS

We are grateful to A Morinibu and K Shinomiya for skilled technical assistance. This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Culture, Sports, Science and Technology, and by a Grant-in-Aid for the 2nd and 3rd Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour, and Welfare, Japan. This study was part of a joint research project, focusing on the development of the basis of technology for establishing a center of excellence for nano-medicine, carried out through Kyoto City Collaboration of Regional Entities for Advancing Technology Excellence (CREATE) assigned by the Japan Science and Technology Agency (JST).

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

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Hypoxia inducible factor-1 influences sensitivity to paclitaxel of human lung cancer cell lines under normoxic conditions

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(Received April 4, 2007/Revised April 27, 2007/Accepted May 1, 2007/Online publication June 29, 2007)

Paclitaxel (PTX) is an anticancer drug that is effective against a wide range of solid tumors. The effect of PTX on two human lung cancer cell lines, PC14PE6 and NCI-H441 cells, was examined in an orthotopically transplanted animal model with an *in vivo* imaging devise. Although PTX effectively suppressed tumor growth and improved survival rate in NCI-H441, it did not influence these in PC14PE6. *In vitro* experiments confirmed that PC14PE6 cells are resistant to PTX under normoxic conditions and that both cell lines were resistant to PTX under hypoxic conditions. It was found that the expression level of endogenous hypoxia inducible factor (HIF)-1α in PC14PE6 is much higher than that in NCI-H441 cells under normoxic conditions. Furthermore, sensitivity to PTX in these cell lines was reversed when HIF-1α expression was decreased by siRNA specific to HIF-1α in PC14PE6 and increased by overexpression of the exogenous *HIF-1α* gene in NCI-H441. These results suggest that HIF-1 influences the PTX sensitivity of these cells. The authors further examined β-tubulin, a target molecule of PTX, with western blotting and immunohistochemical analysis in these cells. The expression level of β-tubulin was comparable in these cells under both normoxic and hypoxic conditions while the distribution of β-tubulin and cell morphology were changed according to HIF-1α expression levels, suggesting that HIF-1 influences the conformation and dynamics of microtubules. These data support the potential development of HIF-1 targeted approaches in combination with PTX, where drug resistance tends to contribute to treatment failure. (*Cancer Sci* 2007; 98: 1394–1401)

PTX is one of the most important agents for first-line chemotherapy in clinical cancer management. It has shown promising response and improved the survival time in cancer patients with non-small cell lung cancer, breast cancer, ovarian cancer, head and neck cancer, and so on.[1] The cellular target for PTX has been identified as the microtubule system, which plays a significant role in mitosis, intracellular transport, cell motility and maintenance of cell shape.[2] PTX arrests the cell cycle in cancer cells via inhibition of microtubule dynamics, in which PTX promotes the assembly of stable microtubules from α- and β-tubulin.[3]

Clinical and experimental studies have shown dramatic heterogeneities in the response of cancer cells to tubulin-binding agents, including PTX. Approximately 20–50% of the human cancers are sensitive at the first treatment of PTX or other microtubule stability agents.[4–6] This means that more than half of cancer cells are resistant to tubulin-binding agents. It has been reported that microtubule-stabilizing and microtubule-destabilizing drugs inhibit HIF-1α accumulation, which leads to suppression of HIF-1 activity and subsequent tumor progression and angiogenesis.[7,8]

Hypoxia is one of the major resistance factors in conventional chemotherapy. Within solid tumors, hypoxic cell chemoresistance was originally attributed to poor drug delivery and to the contention that hypoxic tumor cells are predominantly quiescent. More recently, the contributory role of HIF-1 has been revealed.[9–11] HIF-1 is a heterodimeric transcription factor composed of α and β subunits and is activated in a wide range of cancer cells.[12] HIF-1 increases metabolic adaptation to O_2 deprivation by inducing several decad downstream genes, which in part play a role in tumor progression, including erythropoietin, transferrin, endothelin-1, inducible nitric oxide synthetase, hemeoxygenase 1, and VEGF.[13] The α subunit of HIF-1 has been reported to be an important predictor of tumor progression for several types of solid tumors[14–17] and to be associated with resistance to chemotherapy.[18,19] Although Huang *et al.* have reported that induced HIF-1α expression by $CoCl_2$ could increase the resistance to PTX in an ovarian cell line,[20] a direct correlation between endogenous HIF-1 expression levels and PTX sensitivity has not been described.

In the present paper the authors investigated the direct correlation between the resistance to PTX under normoxic conditions and HIF-1α expression in two human lung cancer cell lines, PC14PE6 and NCI-H441, which are PTX-resistant and -sensitive, respectively, in an *in vivo* orthotopic lung cancer model. Although both cell lines were resistant to PTX under hypoxic conditions *in vitro*, only NCI-H441 showed high sensitivity to PTX under normoxic conditions. The sensitivity of these cell lines to PTX was reversed by changing their HIF-1α expression level using forced expression of the exogenous gene or suppressed expression with siRNA. HIF-1α expression did not influence the protein level of tubulin but did significantly affect cell morphology and tubulin distribution. These data suggest that HIF-1 influences cancer cell sensitivity to PTX by affecting tubulin.

Materials and Methods

Cell cultures and hypoxic treatment. The NCI-H441 cell line was obtained from the ATCC. The PC14PE6 human adenocarcinoma cells were selected from the parental PC14 cell line for the

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Abbreviations: ATCC, American Type Culture collection; EGTA, ethylene glycol bis(βaminoethyl ether)-N,N,N',N'-tetra-acetic acid; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIF, hypoxia inducible factor; HP, horseradish peroxidase; MDR, multidrug resistance; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; P-gp, P-glycoprotein; PTX, paclitaxel; RPMI, Roswell Park Memorial Institute; SDS, sodium dodecyl sulfate; siRNA, small inhibitory ribonucleic acid; SDC, sodium dodecyl chlorate; VEGF, vascular endothelial growth factor.

doi: 10.1111/j.1349-7006.2007.00537.x