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Gene Therapy Strategy for Tumour Hypoxia

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1. Introduction

A characteristic feature of solid tumours is the presence of cells under very low oxygen tensions, hypoxia. These cells, so called hypoxic tumour cells, express a transcription factor, hypoxia-inducible factor 1 (HIF-1), which induces the expression of more than one hundred genes related to angiogenesis, invasion, metastasis, and resistance to conventional treatments such as chemotherapy and radiotherapy. However, because hypoxia is unique to locally advanced malignant tumours, it provides the opportunity to develop tumour-specific targeting strategies. Such an approach has been applied to gene therapy; for example, hypoxia-activated gene therapy using HIF-1-dependent promoters resulted in the selective expression of therapeutic genes and anti-tumour effects with minimum side effects in normal tissues. Here, I review recent advances in the development of cancer gene therapy strategies targeting hypoxic/HIF-1-active tumour cells.

2. A tumour-specific microenvironment, hypoxia, as a therapeutic target

Most human tumours are highly heterogeneous and involve diverse microenvironments. A typical microenvironment seen in solid tumours is hypoxia, low-oxygen conditions under physiological level (Thomlinson & Gray, 1955; Vaupel, Kallinowski, & Okunieff, 1989). Tumour hypoxia is a concern in cancer therapy because it increases the metastatic and angiogenic potential of cancer cells (Erler et al., 2006; Forsythe et al., 1996; Yang et al., 2008) and can render cancer cells resistant to radiation and chemotherapy (Brown & Wilson, 2004; Teicher, 1994; Thomlinson & Gray, 1955).

2.1 What is tumour hypoxia and how does it occur in solid tumours?

A typical feature of cancer cells is an extraordinarily accelerated proliferation caused by the activation of oncogenes and/or disruption of tumour suppressor genes (Hanahan & Weinberg, 2000). It leads to an imbalance in the supply and consumption of O₂ in a solid tumour. This disequilibrium along with the inadequate diffusion of molecular oxygen can cause a dynamic gradient of O₂ content in a solid tumour (Thomlinson & Gray, 1955; Vaupel et al., 1989). Tumour cells proliferate and grow actively only if supplied with enough oxygen and nutrients from tumour blood vessels; therefore, malignant solid tumours grow as a conglomerate of so-called “tumour cords” in each of which a blood vessel is sequentially surrounded with well-oxygenated viable cells (normoxic cells), dormant cells

under low oxygen conditions (hypoxic cells), and dead cells (necrotic cells) Fig. 1(Hall, 1994). Because of the distance that O_2 can diffuse, hypoxic cells exist 70-100 μm from a tumour blood vessel in a tumour cord (Hall, 1994). Hypoxic conditions are usually defined as $< 2\%$ O_2 , and anoxic conditions (severe hypoxia) as $< 0.02\%$ O_2 .

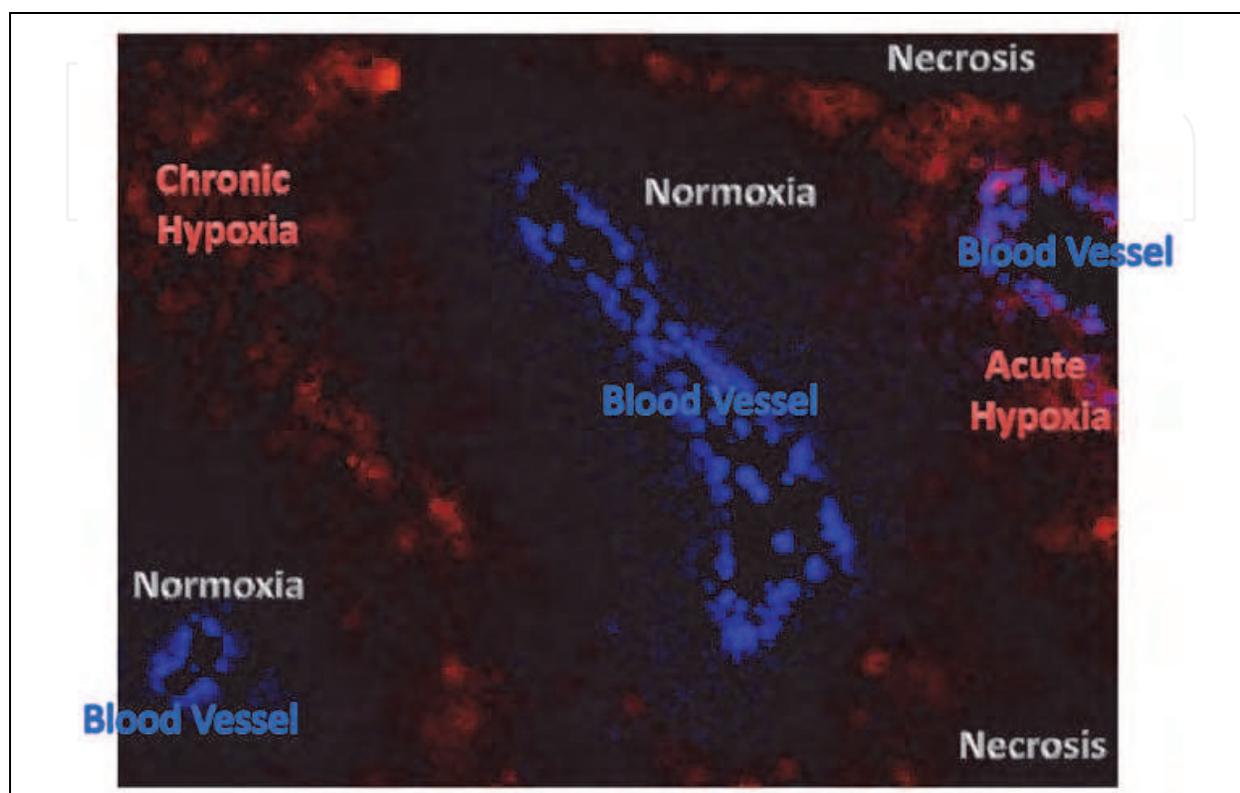


Fig. 1. Spatial relationship between blood vessels and hypoxia in a malignant solid tumour. Chronic hypoxic regions (red) exist 70-100 μm from tumour blood vessels. Acute/cycling hypoxia caused by fluctuations in tumour blood flow occurs proximal to tumour blood vessels.

In addition to areas of chronic hypoxia, malignant tumours contain cancer cells which are temporally exposed to low oxygen conditions for minutes to hours and then reoxygenated (Brown, 1979). This phenomenon, called "acute hypoxia", frequently reoccurs during tumour growth, leading to "cycling hypoxia". The occurrence of acute and cycling hypoxia is attributed to the fact that tumour blood vessels are quite immature and tortuous; and therefore, tumour blood flow fluctuates dramatically during tumour growth (Brown & Wilson, 2004).

2.2 Chemo-resistance of cancer cells under hypoxic conditions

Cancer cells are known to become chemo-resistant in hypoxic regions of locally advanced solid tumours through multiple mechanisms (Kizaka-Kondoh, Inoue, Harada, & Hiraoka, 2003; Teicher, 1994). First, because hypoxic regions occur far from functional vasculatures, the diffusion and delivery of most anticancer drugs are not extensive enough to have a cytotoxic effect (Durand, 1994; Hicks et al., 2006; Tannock, 1998). Second, the cytotoxicity of some anticancer drugs is known to depend on molecular oxygen. For example, bleomycin is reported to chelate metal ions, produce a pseudoenzyme that reacts with oxygen and

generates superoxide and hydroxide free radicals, and then cleave DNA. Therefore, the cytotoxic effect of the drug dramatically decreases in the absence of O₂ (Batchelder, Wilson, Hay, & Denny, 1996; Teicher, Lazo, & Sartorelli, 1981). Third, alkylating agents and antimetabolites are also less effective under hypoxic conditions. These kinds of drugs are the most effective against highly proliferating cancer cells, and therefore, hypoxic tumour cells, which are known to be dormant/less proliferating, can tolerate them (Tannock, 1968). Fourth, hypoxia upregulates the expression of genes involved in drug resistance, including the gene for p-glycoprotein (Comerford et al., 2002; Wartenberg et al., 2003). Finally, there is evidence that hypoxia can enhance genetic instability in tumour cells (N. Chan et al., 2008), thus allowing a more rapid development of drug resistance.

2.3 Radio-resistance of cancer cells under hypoxic conditions

Ionizing radiation produces DNA damage, such as DNA double/single strand breaks, DNA base damage, and DNA-DNA and DNA-protein crosslinks (Hall, 1994). The presence or absence of molecular oxygen influences the damage and death of cancer cells (Brown & Wilson, 2004; Thomlinson & Gray, 1955). This phenomenon, the so-called oxygen effect, was first identified in 1912 with the observation that the skin reaction to a radium applicator dramatically decreased when the applicator was pushed tightly onto the skin and consequently decreased blood flow there. The breakthrough linking the effect of oxygen with radioresistance of cancer cells was made by Thomlinson and Gray in 1955 (Thomlinson & Gray, 1955). They proposed that oxygen levels decreased in a solid tumour through successive layers of cancer cells distal to blood vessels, and cancer cells a distance of about 10 cell diameters from vessels are viable but radioresistant. Actually, cancer cells become 2-3 times more radioresistant under hypoxic conditions than normoxic conditions (Brown & Wilson, 2004).

The hypoxia-mediated radioresistance is attributed to both chemical and biological mechanisms. Ionizing radiation induces ionization in or close to the genomic DNA of target cells and produces radicals (Brown & Wilson, 2004). The DNA radicals are subjected to oxidation in the presence of oxygen, leading to fixation of the damage. In the absence of oxygen, however, the DNA radicals are reduced by compounds containing sulfhydryl groups (SH groups), which restore the DNA to its original form. Therefore, DNA damage, especially irreparable double stranded breaks, is significantly less severe in the absence of molecular oxygen. Biological mechanisms are also important. It has been elucidated that hypoxic stimuli trigger changes in both the "DNA damage repair pathway" (Bindra, Crosby, & Glazer, 2007) and the "cell death/survival signaling pathway". Moreover, recent advances in molecular and cellular biology revealed an important role for a transcription factor, hypoxia-inducible factor 1 (HIF-1), in tumour radioresistance (*see* Section 3 for details) (Harada & Hiraoka, 2010).

2.4 Increase in metastatic and angiogenic potential under hypoxic conditions

In addition to mediating resistance to conventional treatments, hypoxia is known to increase the metastatic and angiogenic potential of tumour cells. Cancer patients with relatively more hypoxic regions have a tendency to suffer from distant metastasis as well as local recurrence regardless of whether the initial treatment is surgery or radiation therapy (Brizel et al., 1996). Recent molecular biological analyses have revealed that hypoxia stimulates the expression of a number of genes involved in metastatic cascades, such as lysyl oxidase and the chemokine receptor, CXCR4, osteopontin (D. A. Chan & Giaccia, 2007; Erler et al., 2006;

Rofstad, 2000). Also, cancer cells under hypoxic conditions trigger angiogenesis in order to improve surrounding conditions and obtain enough oxygen and nutrients for their survival (Folkman, 1971). HIF-1 is known to play a pivotal role in the hypoxia-mediated increase in both the metastatic and angiogenic potential of cancer cells.

3. Hypoxia-inducible factor 1 (HIF-1)

Molecular and cellular biological research has identified HIF-1 as an important transcription factor in hypoxia-mediated angiogenesis, metastasis, and resistance to chemo/radiotherapy.

3.1 Regulation of HIF-1 expression and activity

HIF-1 is a heterodimeric transcription factor composed of alpha (HIF-1 α) and beta (HIF-1 β /ARNT) subunits (Wang, Jiang, Rue, & Semenza, 1995). Its hypoxia-dependent activity is mainly regulated through the stabilization and modification of the HIF-1 α subunit (Fig. 2).

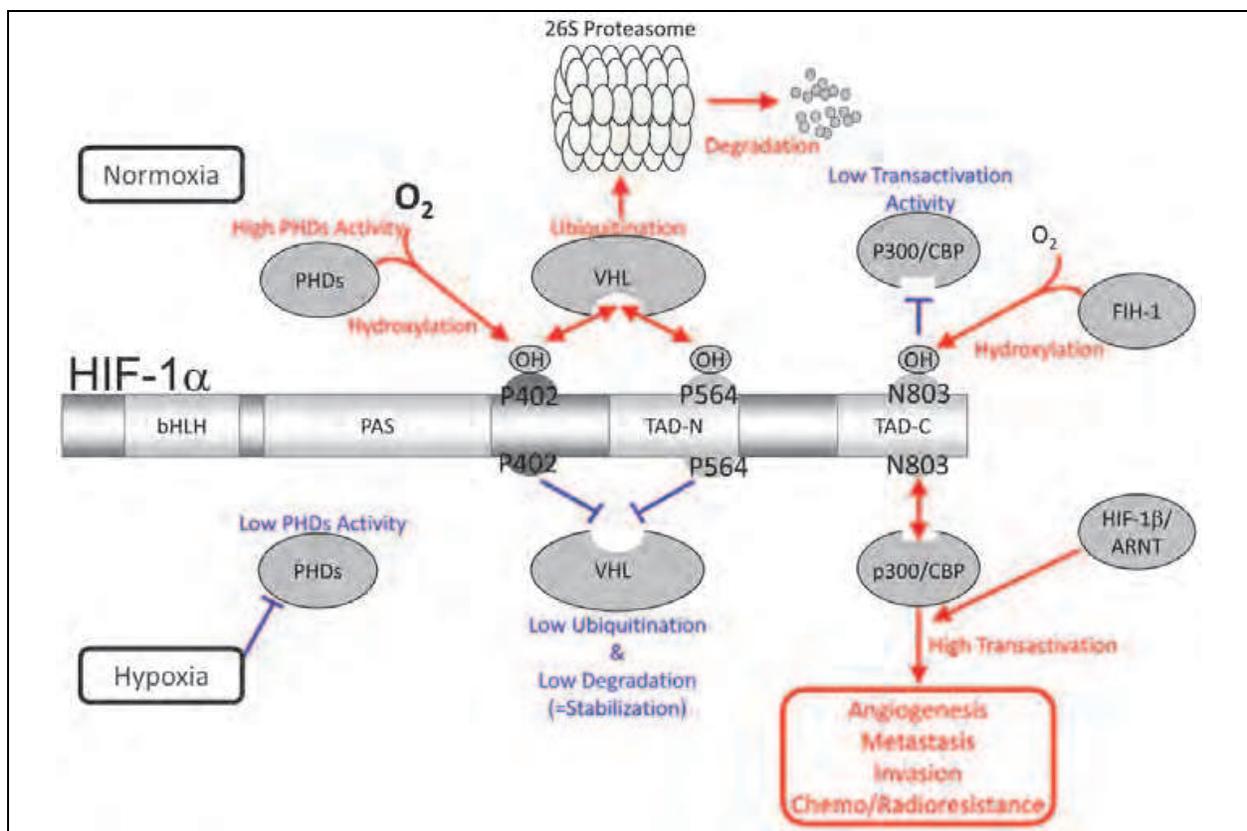


Fig. 2. Molecular mechanism behind the activation of HIF-1 under Hypoxic conditions.

The best-characterized regulatory mechanism is that modulating HIF-1 α 's stability. Under well-oxygenated normoxic conditions, prolyl hydroxylation (by prolyl hydroxylases [PHDs]) and subsequent ubiquitination (by von-Hippel Lindau (VHL)-containing E3 ubiquitin-protein ligase) of the oxygen-dependent degradation (ODD) domain of HIF-1 α leads to rapid degradation of HIF-1 α with a half life of 5-8 min. Consequently, HIF-1 is inactive under normoxic conditions (Berra, Roux, Richard, & Pouyssegur, 2001; Hirota & Semenza, 2005; Jaakkola et al., 2001; Maxwell et al., 1999; Semenza, 2001). On the other hand, under oxygen-deprived hypoxic conditions, HIF-1 α becomes stable because oxygen-

depletion directly decreases the PHDs' activity (Jaakkola et al., 2001). Then, HIF-1 α interacts with HIF-1 β , forms a heterodimer, HIF-1 (Wang et al., 1995), binds to its cognate DNA sequence, the hypoxic-responsive element (HRE), and finally induces the expression of various genes related to angiogenesis, metastasis, glycolysis and so on (D. A. Chan & Giaccia, 2007; Erler et al., 2006; Forsythe et al., 1996; Kim, Gao, & Dang, 2007; Rofstad, 2000). In addition to the regulation of HIF-1 α 's stability, another post-translational modification of HIF-1 α is known to function in the regulation of the transactivational activity of HIF-1. Under normoxic conditions, factor inhibiting HIF-1 (FIH-1) becomes active and hydroxylates an asparagine residue (N803) of HIF-1 α (Hirota & Semenza, 2005; Mahon, Hirota, & Semenza, 2001; Semenza, 2001). The asparaginyl hydroxylation blocks the interaction of HIF-1 α with the transcriptional co-factor p300 and CBP, resulting in the suppression of HIF-1's transactivational activity. Because oxygen is a substrate of FIH-1 as well as PHDs, HIF-1's transactivational activity is restored under oxygen-deprived hypoxic conditions.

3.2 Function of HIF-1 in cancer cells

In cancer cells, HIF-1 plays pivotal roles in the adaptation to (metabolic reprogramming), evasion from (invasion and metastasis), and improvement of (angiogenesis) severe hypoxic conditions (D. A. Chan & Giaccia, 2007; Erler et al., 2006; Forsythe et al., 1996; Semenza, 2007, 2008). Concerning angiogenesis in locally advanced malignant tumours, an up-regulation of HIF-1 activity caused by intratumoural hypoxia is associated with the overexpression of vascular endothelial growth factor (VEGF), a glycoprotein responsible for angiogenesis and vasculogenesis (Forsythe et al., 1996). Concerning the metabolic reprogramming of cancer cells, HIF-1 induces the expression of genes encoding glucose transporters and glycolytic enzymes to facilitate glycolysis (Semenza, 2009; Wood et al., 1998). At the same time, HIF-1-dependent genes decrease both mitochondrial metabolism (Fukuda et al., 2007; Semenza, 2007) and mitochondrial mass (Semenza, 2008; H. Zhang et al., 2008). These functions of HIF-1 are responsible for both the efficient production of ATP even under oxygen-deprived conditions and the decrease in the cytotoxic reactive oxygen species (ROS) produced through incomplete oxidative phosphorylation under hypoxic conditions (Fukuda et al., 2007; Semenza, 2007, 2009). Concerning invasion and metastasis, HIF-1 is known to trigger various pathways including epithelial-mesenchymal transition (EMT) and expression of the Met protooncogene and lysyl oxidase, which function in tumour metastasis (Erler et al., 2006; Pennacchietti et al., 2003).

3.3 Function of HIF-1 in tumour radioresistance

Preclinical studies have found that inhibition of intratumour HIF-1 activity by a pharmacological HIF-1 inhibitor, YC-1, or by a dominant negative mutant of HIF-1 α and the knockdown of HIF-1 α expression by short hairpin RNA or short interfering RNA significantly delayed tumour growth after radiation (Harada et al., 2009; Moeller, Cao, Li, & Dewhirst, 2004; Moeller et al., 2005; X. Zhang et al., 2004). It was also confirmed through clinical studies that HIF-1 α expression correlates with a poor prognosis after radiation therapy (Aebersold et al., 2001; Irie, Matsuo, & Nagata, 2004; Ishikawa et al., 2004). These results imply that HIF-1 has a certain biological function to increase tumour radioresistance. Actually, HIF-1-mediated radioresistance has been recently revealed: 1) radiation activates HIF-1 in a solid tumour, 2) HIF-1 induces the expression of VEGF, 3) VEGF protects endothelial cells from the cytotoxic effects of radiation, and 4) the radio-protected tumour

blood vessels assure the supply of oxygen and nutrients to tumour cells and promote tumour growth (Harada et al., 2009; Moeller et al., 2004; Zeng et al., 2008).

4. Development of gene therapy strategies targeting tumour hypoxia

Because hypoxic/HIF-1-active cells are known to mediate tumour malignancy and resistance to conventional treatments, and because hypoxia has been recognized as a tumour-specific microenvironment, recent studies have tried to exploit hypoxic cells as targets for cancer therapy (Brown & Wilson, 2004; Harris, 2002; Semenza, 2003). Dachs *et al.* were the first to apply this concept to gene therapy (Dachs, Patterson, et al., 1997). Since they demonstrated the effectiveness of hypoxia-specific gene therapy strategy using the HIF-1/HRE system, extensive efforts have been devoted to developing genetically engineered hypoxia-responsive promoters.

4.1 Development of HIF-1-dependent promoters

Various HREs, such as murin phosphoglycerate kinase-1 (PGK-1) HRE, human enolase (ENO) HRE, murin lactate dehydrogenase (mLDH-A) HRE, human erythropoietin (EPO) HRE, and human VEGF HRE, have been used to develop artificial hypoxia-responsive promoters (Table 1) (Binley, Iqball, Kingsman, Kingsman, & Naylor, 1999; Boast et al., 1999; Dachs, Patterson, et al., 1997; Harada et al., 2007; Rinsch et al., 1997; Shibata, Akiyama, Noda, Sasai, & Hiraoka, 1998; Shibata, Giaccia, & Brown, 2000). The number of HREs and combination with the basal promoter influence the hypoxia/HIF-1-responsiveness of each HRE-containing promoter (Table 1).

Above all, the combination of five repeats of a HRE derived from the human VEGF promoter and the human cytomegarovirus (CMV) minimal promoter (mp), the so-called “the 5HRE promoter“, showed intense hypoxia-responsiveness and exhibited a more than 500-fold increase in luciferase activity in response to hypoxic stimuli (Shibata et al., 2000). Moreover, the absolute level of luciferase activity from the 5HRE promoter under hypoxic conditions reached the same level as that from the constitutively active CMV-driven promoter under normoxic conditions (Shibata et al., 2000).

However, the 5HRE promoter still has problems relating to the development of gene therapy. It shows a certain level of unwanted gene expression even when oxygen is available under normoxic conditions (Harada et al., 2007), which would cause high basal expression of therapeutic genes and result in side effects in well-oxygenated normal tissues. In order to decrease leakage under normoxic conditions, I and my colleagues came up with the idea of utilizing the ODD domain of HIF-1 α . We fused the coding sequence of the ODD domain to that of luciferase, and inserted the fusion gene downstream of the 5HRE promoter. The resultant *5HREp-ODD-luc* gene showed little leakage under normoxic conditions. Leakage from the conventional *5HREp-luc* gene was 1.4×10^3 (arbitrary units), on the other hand, that from the novel *5HREp-ODD-luc* gene was just 1.5×10^1 (arbitrary units), almost the same as the background level. Moreover, the oxygen-dependent destabilizing effect of the ODD domain contributed to an increase in the hypoxia-responsiveness to about 4.7×10^4 .

The potential of hypoxia/HIF-1-dependent promoters *in vivo* has been proved through immunohistochemical analyses and optical imaging experiments. The human melanoma cell line, Be11, was stably transfected with a plasmid expressing a derivative of EGFP, d2EGFP, under the control of the 5HRE promoter, and transplanted into immunodeficient nude mice

HRE	Basal Promoter	Reporter Gene	Induction Ratio	References
3 × mPGK-1	mPGK-1	CD2	1.4-1.9	Dachs, 1997
3 × mPGK-1	minHSV TK	CD2	2.2-2.33	
3 × mPGK-1	9-27 gene	CD2	2.2-4.1	
mPGK-1	mPGK-1	hEPO	2.7	Rinsch, 1997
hVEGF	hVEGF	luciferase	3.3-8.5	Shibata, 1998
hEPO	SV40	luciferase	2-5	
5 × hVEGF	hVEGF	luciferase	20	
5 × hVEGF	hVEGF+ minE1b	luciferase	44	
3 × hENO	SV40	luciferase	120	Boast, 1999
3 × hENO	SV40	luciferase	63	
2 × mLDHA	SV40	luciferase	81	
4 × mLDHA	SV40	luciferase	65	
4 × hEPO	SV40	luciferase	255	
3 × hENO	SV40	luciferase	146	
3 × mPGK-1+ VEGF 3' UTR	SV40	luciferase	300	
mPGK-1	SV40	β-gal	8.5-50	Binley, 1999
5 or 10 × hVEGF	SV40	luciferase	54-57	Shibata, 2000
5 or 10 × hVEGF+ 5' VEGF UTR	SV40	luciferase	23-27	
5-10 × hVEGF+ 5' VEGF UTR	E1b	luciferase	56-60	
5 × hVEGF+ 5' VEGF UTR	E1b	luciferase	131	
5 × hVEGF+ 5' VEGF UTR+ 3' VEGF UTR	E1b	luciferase	193	
5 × hVEGF	hCMV-mp	luciferase	524	
5 × hVEGF	hCMV-mp	luciferase	47,000	

Table 1. Genetically engineered hypoxia-responsive promoters.

(Liu et al., 2005). Resultant tumour xenografts showed heterogeneous, partition-dependent and weak green fluorescence. Immunohistochemical analyses confirmed that d2EGFP-positive cells were located at the boundary between well-oxygenated viable regions and necrotic regions, which were stained with a hypoxia marker, pimonidazole (Raleigh et al., 1998). When human cervical cancer cells, HeLa cells, transfected with the *5HREp-luc* or *5HREp-ODD-luc* gene were transplanted into nude mice, the resultant xenografts showed intense bioluminescence after the tumour-bearing leg was ligated and the blood flow to the xenograft decreased (Harada, Kizaka-Kondoh, & Hiraoka, 2005; Harada et al., 2007).

4.2 Hypoxia-targeted gene-directed enzyme/prodrug therapy using a HIF-1-dependent promoter

To exploit tumour hypoxia/HIF-1 active cells as a tumour-specific therapeutic target, several approaches have been examined including (1) hypoxia-targeting using hypoxia/HIF-1-responsive promoters combined with Gene-Directed Enzyme Prodrug

Therapies (GDEPT) (Greco, Marples, Joiner, & Scott, 2003; Liu, Harada, Ogura, Shibata, & Hiraoka, 2007; Ogura et al., 2005; Patterson et al., 2002; Shibata, Giaccia, & Brown, 2002), and (2) hypoxia-specific replication of adenovirus (Hernandez-Alcoceba, Pihalja, Qian, & Clarke, 2002; Post & Van Meir, 2003) (Table 2).

Delivery System	Hypoxia-specific Strategy	Result	Reference
NA (Transfected cancer cell line)	GDEPT: 5 x VEGF-HRE-containing promoter driven NTR gene expression in HT1080 cells and i.p. injection of the anticancer prodrug CB1954.	Significant tumour growth delay	Shibata, 2002
NA (Transfected cancer cell line)	GDEPT: 5 x VEGF-HRE-containing promoter-driven HSV-TK gene expression and i.p. injection of GCV	Tumour growth suppression	Ogura, 2005
NA (Transfected cancer cell line)	GDEPT: PGK-1 HRE/SV40 chimeric promoter-driven P450R gene expression and i.p. injection of the 2-nitroimidazole bioreductive prodrug, RB6145	Enhanced Radiotherapy	Patterson, 2002
Adenovirus	GDEPT: optimized hypoxia response promoter using PGK-1 HRE-driven human cytochrome P450 (CYP2B6) gene expression and i.p. injection of cyclophosphamide	Delay in tumour growth	Binley, 2003
Adenovirus	3XHRE/5XERE-regulated E1A expression and hTERT (AdeHT2) or E2F-1 (AdeHE2F) promoter-regulated E4 expression.	Tumour growth suppression and regression	Hernandez-Alcoceba, 2002
Adenovirus (intratumoural injection)	GDEPT: 5 x VEGF-HRE-containing promoter-driven bacterial cytosine deaminase (BCD) gene expression and i.p. injection of 5-FC.	Tumour growth suppression and enhanced radiotherapy	Liu, 2007
Adenovirus	human VEGF promoter-driven BAX gene expression	induction of apoptosis and suppression of cell growth in vitro	Kaliberov, 2002

Table 2. Gene therapy strategies for tumour hypoxia examined so far.

NA, not applicable; HSV-TK, the herpes simplex virus thymidine kinase; GCV, ganciclovir; GDEPT, Gene-Directed Enzyme Prodrug Therapy; NTR, nitroreductase; CRAD, conditionally replicative adenoviruses; ER, estrogen receptor; ERE, estrogen response element; hTERT, human telomerase reverse transcriptase; CD, cytosine deaminase; VEGF, Vascular Endothelial Growth Factor, 5-FU, 5-fluorouracil; 5-FC, 5-flucytosine;

4.2.1 Gene-directed enzyme prodrug therapy (GDEPT) with HIF-1-dependent promoters

GDEPT involves the delivery to target cells of a foreign gene, which is non-toxic but activates prodrugs to toxic agents and induces anti-tumour effects (Fig. 3 Dachs, Dougherty, Stratford, & Chaplin, 1997; Greco et al., 2003). HIF-1 activity is detected at high levels in hypoxic tumour cells but generally not in normal tissues as mentioned above; therefore, HIF-1-dependent promoters provide a chance to accomplish tumour-specific GDEPT. The target-specificity and therapeutic effects of GDEPT with HIF-1-dependent promoters have been examined in experimental tumour systems either by administering viral vectors (Binley et al., 2003; Liu et al., 2007) or by using unique tumour xenografts prepared by transplanting cancer cells which express a prodrug-activating enzyme under the control of a HIF-1-dependent promoter (Ogura et al., 2005; Patterson et al., 2002; Shibata et al., 2002). Examples of enzyme/prodrug combinations in GDEPT are the bacterial nitroreductase (NTR)/anticancer prodrug CB1954, the cytochrome P450 reductase (P450R)/RSU1069, the herpes simplex virus thymidine kinase (HSV-TK)/ganciclovir (GCV), and the bacterial cytosine deaminase (BCD)/anti-herpes viral agent 5- fluorocytosine (5-FC).

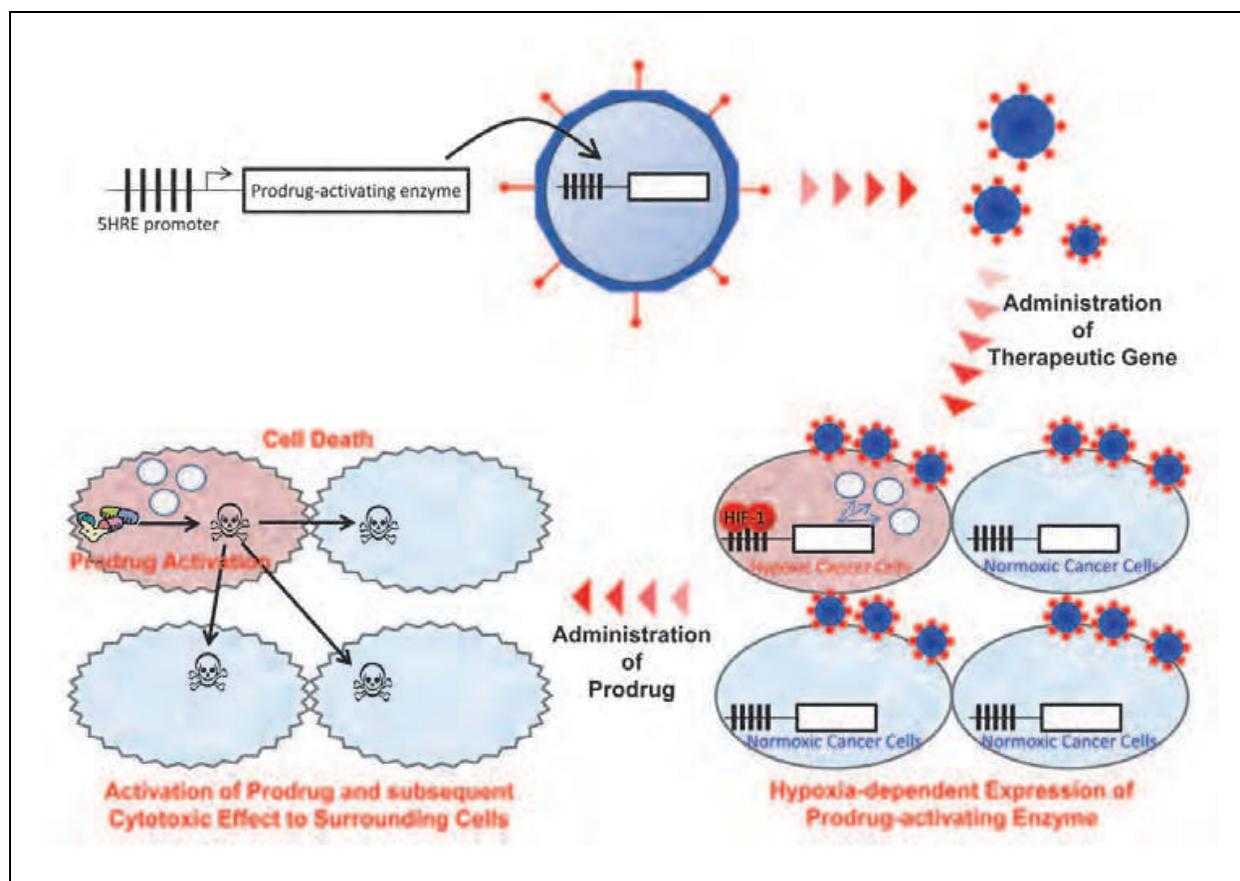


Fig. 3. Concept of GDEPT targeting tumour hypoxia.

Using HREs derived from the VEGF promoter, Shibata *et al.* generated vectors expressing a bacterial NTR gene in a HIF-1-dependent manner, and established stable transfectants of a human fibrosarcoma cell line, HT1080 (Shibata et al., 2002). Hypoxia-induced expression of the NTR protein correlated with increased sensitivity of HT1080 cells to the prodrug CB1954 (Shibata et al., 2002). Growth delay assays with established tumour xenografts derived from

the same cells showed that significant antitumour effects were achieved with intraperitoneal injections of CB1954 (Shibata *et al.*, 2002). In addition, respiration of 10% O₂ increased the hypoxic fraction in the tumour xenograft *in vivo* and enhanced the antitumour effects (Shibata *et al.*, 2002).

A one-electron reductase, such as P450R, can be used for prodrug-activation. Patterson *et al.* reported that selective hypoxia-targeting could be accomplished by using an optimized PGK-1 HRE/SV40 chimeric promoter to regulate the expression of P450R. HT1080 cells stably transfected with the gene produced a 3.4-fold increase in P450R activity as a result of anoxic incubation, leading to a 30-fold enhancement of the cytotoxicity of the 2-nitroimidazole bioreductive prodrug, RSU1069 (Patterson *et al.*, 2002).

As for HSV-TK/GCV gene therapy, Ogura *et al.* demonstrated that promoters containing 5 copies of the VEGF HRE assured the expression of the HSV-TK gene in a HIF-active renal cell carcinoma, resulting in GCV-dependent tumour growth suppression (Ogura *et al.*, 2005). An important point of their study is that HIF-dependent HSV-TK/GCV gene therapy can target not only hypoxic but also normoxic renal cell carcinoma (RCC) cells because the VHL gene, which triggers the destruction of HIF-1 α and HIF-2 α during normoxia, is inactive in 33–57% of sporadic clear-cell RCCs, which accounts for 75% of RCCs.

Binley *et al.* used an optimized hypoxia-responsive promoter (OBHRE) with the PGK-1 HRE and investigated hypoxia-targeted gene expression *in vivo* in the context of an adenoviral vector (Binley *et al.*, 2003). The OBHRE promoter showed limited activity in the liver or spleen such that expression was 1000-fold lower than that driven by the strong CMV/IE promoter (Binley *et al.*, 2003). However, in the context of the tumour microenvironment, the OBHRE promoter achieved expression levels comparable to that of the CMV/IE promoter. Moreover, they showed that an adenovirus expressing the human cytochrome P450 (CYP2B6) regulated by the OBHRE promoter delays tumour growth in response to the prodrug cyclophosphamide (CPA) (Binley *et al.*, 2003).

As for CD/5-FC gene therapy, Liu *et al.* constructed an adenoviral vector in which the 5HRE promoter was responsible for the HIF-1-dependent expression of CD (Liu *et al.*, 2007). Administration of the adenovirus resulted in the expression of CD in hypoxic regions of solid tumours, leading to 5-FC-dependent tumour growth suppression and enhancement of the therapeutic effect of radiation therapy (*See* Section 4.4 for details about the radio-enhancing effect).

In general, GDEPT is thought to have two advantages: amplification of its therapeutic effect and a bystander therapeutic effect. The former is due to the ability of each prodrug-activating enzyme to activate a number of prodrug molecules. The latter advantage results from an extension of the killing effects of the activated/converted drug to surrounding cancer cells, which don't express the therapeutic gene and don't convert the prodrug to the active anticancer drug. Therefore, even if systemic delivery of the therapeutic genes is not effective Fig. 3, tumour eradication may still be achieved.

4.2.2 Other gene therapy strategies targeting tumour hypoxia

4.2.2.1 Gene therapy using a cytotoxic gene and HIF-1-dependent promoter

An alternative GDEPT uses cytotoxic proteins instead of prodrug-activating enzymes expressed in a HIF-1-dependent manner (Table 2). Kaliberov *et al.* prepared an adenovirus, AdVEGFBAX, which expressed an inducer of apoptosis, BAX, under the control of a VEGF promoter (Kaliberov *et al.*, 2002). They confirmed the potential therapeutic application of VEGF promoter-driven cancer-specific expression of the pro-apoptotic Bax gene.

4.2.2.2 Gene therapy using a hypoxia/HIF-dependent oncolytic adenovirus

The lytic cycle of adenoviruses is known to result in the death of infected cells, and thus this property has been exploited as a therapeutic strategy against cancer. Post and Van Meir have developed a hypoxia/HIF-dependent replicative adenovirus (HYPR-Ad) that exhibits HIF-1-dependent E1A expression and conditional cytolysis of hypoxic cells but not normoxic cells (Post & Van Meir, 2003). This is the first evidence that an attenuated oncolytic adenovirus that selectively lyses hypoxic tumour cells can be generated.

4.3 Side effect of hypoxia-targeting gene therapies

To measure the damage to normal tissue after gene therapy targeting tumour hypoxia, Binley *et al.* evaluated the activity of lactate dehydrogenase (LDH) as an indicator of liver dysfunction after their hypoxia-responsive thymidine kinase/ganciclovir (TK/GCV) suicide gene therapy (Binley *et al.*, 2003). Hypoxia-dependent TK expression and subsequent 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, meaning an increased therapeutic window for cytotoxic cancer gene therapies.

Liu *et al.* observed no obvious side effects after hypoxia-targeting gene therapy with a 5HRE promoter-mediated BCD/5-FC strategy (Liu *et al.*, 2007). On the other hand, after the Ad/EFp-BCD/5-FC treatment, which constitutively expresses BCD regardless of surrounding oxygen conditions, they observed significant weight loss and severe diarrhea (Liu *et al.*, 2007). These results strengthen the argument that we can exploit tumour hypoxia as a tumour-specific target of cancer gene therapy, and that hypoxia/HIF-1-dependent therapeutic gene expression helps to avoid side effects in normal tissues.

4.4 Improvement of the effect of radiotherapy by hypoxia/HIF-1-targeting gene therapies

As tumour hypoxia and HIF-1 activity are responsible for tumour radioresistance (Aebbersold *et al.*, 2001; Harada *et al.*, 2009; Irie *et al.*, 2004; Ishikawa *et al.*, 2004; Moeller *et al.*, 2004; Moeller *et al.*, 2005; X. Zhang *et al.*, 2004), the specific targeting of tumour hypoxia and/or HIF-1 activity by gene therapy strategies may improve the efficacy of radiotherapy. Actually, Patterson *et al.* demonstrated that the therapeutic effect of radiation can be enhanced by a hypoxia-targeting gene therapy strategy (Patterson *et al.*, 2002). They transfected HT1080 cells with a hypoxia-regulated expression vector encoding the human P450 reductase (HRE-P450R). Xenografts of HRE-P450R and empty vector transfectants had comparable hypoxic fractions and were refractive to a single dose of radiotherapy of up to 15 Gy. However, combining a prodrug RSU1069 with a reduced dose of radiotherapy (10 Gy) cured 50% of mice bearing HRE-P450R xenografts by 100 days after the treatment. On the other hand, one hundred percent mortality was observed by day 44 in the empty vector control xenografts treated using the same protocol.

Liu *et al.* treated HeLa tumour xenografts with adenovirus-mediated hypoxia-targeting cytosine deaminase gene therapy (Ad/5HREp-BCD/5-FC) and/or radiotherapy (IR), and carried out growth delay assays (Liu *et al.*, 2007). They intentionally chose a low dose of Ad/5HREp-BCD/5-FC, which had minimal effects on the tumour growth rate compared to that after sham-treatment. Combined with IR, the gene therapy strikingly suppressed tumour growth as compared to radiotherapy alone. The period taken for tumour growth to

increase two-fold from the initial volume (tumour growth doubling time: TGDT) was 13.2 ± 5.6 days after gene therapy alone, which is not significantly longer than that after sham-treatment (8.2 ± 3.1 days). On the other hand, the combination of gene therapy with radiotherapy prolonged the TGDT to 47.2 ± 16.8 days, which was about 2.4-fold longer than that after radiotherapy alone (19.4 ± 4.8 days). Similar results were observed after fractionated irradiation ($3 \text{ Gy} \times 5$ fractions). The TGDT after gene therapy alone was 13.0 ± 4.4 days, which is not significantly longer than that after sham treatment (9.8 ± 5.8 days). On the other hand, the TGDT after the fractionated radiotherapy was 17.0 ± 3.7 days, which was significantly delayed by the combination with the gene therapy to 43.3 ± 23.8 days. These results also lead to a conclusion that hypoxia-targeting gene therapy combined with radiotherapy is a promising approach to cancer treatment.

5. Conclusion

Cancer cells under hypoxic conditions/HIF-1-active cancer cells have been recognized as crucial and excellent targets for cancer therapy not only because they mediate tumour malignancy and resistance to conventional treatments but also because they are only seen in malignant tumours, not in normal tissues. Several approaches have been used to target these cell populations; hypoxia-targeting using hypoxia-responsive promoters combined with GDEPT, and hypoxia-specific replication of adenovirus as well as hypoxic cytotoxins and HIF-1 inhibitors. Hypoxia/HIF-1-targeting gene therapy is a promising tumour-specific approach with few side effects in normal tissues, and has the potential to enhance the effect of radiation therapy. Some approaches are now in clinical trials and are expected to lead to breakthroughs in cancer therapy.

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This book aims at providing an up-to-date report to cover key aspects of existing problems in the emerging field of targets in gene therapy. With the contributions in various disciplines of gene therapy, the book brings together major approaches: Target Strategy in Gene Therapy, Gene Therapy of Cancer and Gene Therapy of Other Diseases. This source enables clinicians and researchers to select and effectively utilize new translational approaches in gene therapy and analyze the developments in target strategy in gene therapy.

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