Real-time Imaging of Hypoxia-inducible Factor-1 Activity in Tumor Xenografts

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Hypoxia/Hypoxia-inducible factor-1 (HIF-1)/Hypoxia-response element (HRE)/Pimonidazole/Destabilized Enhanced GFP (d2EGFP).

Hypoxia-inducible factor-1 (HIF-1) is responsible for various gene expressions related to tumor malignancy, such as metastasis, invasion and angiogenesis. Therefore, monitoring HIF-1 activity in solid tumors is becoming increasingly important in clinical and basic studies. To establish a convenient system for visualizing HIF-1 activity in tumor xenografts, we employed a promoter consisting of five copies of hypoxia response elements (5HRE), whose activity depends on HIF-1, and used a derivative of green fluorescence protein (d2EGFP) as a reporter gene. A human melanoma cell line, Be11, which contains the 5HRE-d2EGFP gene, showed fluorescence in response to hypoxia. The fluorescent intensity correlated inversely with the surrounding oxygen tension, and was time-dependent for the hypoxic treatment. Reoxygenation resulted in a rapid decrease in fluorescence due to the signal sequence for protein degradation encoded in d2EGFP, which enabled monitoring of HIF-1 activity in real-time. Heterogeneous fluorescence was observed in the solid tumor of a non-sacrificed tumor-bearing mouse. Immunohistochemical analysis confirmed that d2EGFP-expressing regions overlapped with the ones stained with a hypoxia marker, pimonidazole. These results suggest that the 5HRE-d2EGFP gene is suitable for the real-time imaging of HIF-1-activating cells \textit{in vivo}, due to the short half-life of the d2EGFP protein as well as the specificity of the 5HRE promoter for HIF-1 activity.

INTRODUCTION

The rapid proliferation and high metabolic demands of cancer cells cause a reduction in oxygen tension to below physiological levels, which is a typical feature of solid tumors and is known as tumor hypoxia.\textsuperscript{1,2} In hypoxic tumor cells, a transcriptional factor, hypoxia-inducible factor-1 (HIF-1), induces various kinds of gene expression related to angiogenesis\textsuperscript{3} and glycolysis\textsuperscript{4}, and appears to play a critical role in the development of invasive and metastatic properties.\textsuperscript{5} Moreover, HIF-1 activity is associated with the resistance of tumor cells against radiotherapy as well as chemotherapy\textsuperscript{6,7}. For example, it has been demonstrated that ionizing radiation (IR) treatment up-regulates HIF-1 activity and induces vascular endothelial growth factor (VEGF) gene expression in tumor cells, which then leads to radioresistance of endothelial cells and eventually to radioresistance of the tumor cells themselves.\textsuperscript{8} In addition, significant associations between HIF-1 activity and patient mortality have been reported in clinical studies of brain\textsuperscript{9}, breast\textsuperscript{10,11}, cervix\textsuperscript{12}, oropharynx\textsuperscript{13}, ovarian cancer\textsuperscript{14}, etc.

HIF-1 is a heterodimeric transcriptional factor composed of α subunit (HIF-1α) and β subunit (HIF-1β), which is also known as ARNT (aryl hydrocarbon receptor nuclear translocator).\textsuperscript{15} HIF-1α expression is regulated in an oxygen-dependent manner at the post-translational level and is responsible for the regulation of HIF-1 activity.\textsuperscript{16} Under aerobic conditions, the proline residues, Pro-402 and Pro-564, in the oxygen dependent degradation (ODD) domain of HIF-1α protein are hydroxylated by prolyl hydroxylase-domain protein 1-3 (PHD1-3).\textsuperscript{17,18} The modified HIF-1α protein is recognized as a target for ubiquitylation by E3 ubiquitin-protein ligases containing the von Hippel-Lindau tumor suppressor protein (pVHL).\textsuperscript{17,18} Consequently, the ubiquitylated HIF-1α is degraded by the 26S proteasome.\textsuperscript{17,18} On the other hand, the rate of prolyl hydroxylation decreases under

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hypoxic condition and VHL cannot recognize HIF-1α as a target, resulting in a reduced rate of degradation.17,18) The stabilized HIF-1α interacts with the constitutively expressed HIF-1β protein and induces the expression of certain genes such as VEGF9),22) glycolytic enzyme23) and erythropoietin.19) The induction of these genes is triggered by the interaction of HIF-1 with its cognate DNA recognition site, that is, the hypoxia-response element (HRE).1,20) Previously, Shibata et al. developed an artificial hypoxia-inducible promoter system using five copies of HRE (5HRE) derived from the VEGF promoter and the human cytomegalovirus minimal promoter (CMVmp), and demonstrated that the 5HRE promoter increased gene expression by a factor of more than 500 under hypoxic conditions in vitro.21,22) In the process of developing radiosensitziers, 2-nitroimidazole derivatives have been found to accumulate in hypoxic cells of solid tumors. Consequently, some methods to obtain quantitative or qualitative data about tumor hypoxia have been developed, and suitably labeled 2-nitroimidazole analogues have been utilized to develop a variety of non-invasive monitoring systems for tumor hypoxia, e.g. magnetic resonance spectroscopy (MRS),23,24) positron emission tomography (PET),25) and single photon electron capture tomography (SPECT).25) Pimonidazole, a well-known hypoxia marker, is also a 2-nitroimidazole derivative and has been developed for the immunohistochemical analysis of tumor specimens.26,27) Polarographic microelectrodes can directly record the oxygen status of tumor tissues and have therefore been widely used in clinical practice for cancer patients.27) Although all of these methods yield detailed information about tumor hypoxia, they do not accurately reflect HIF-1 activity because HIF-1 activation is caused by not only intratumoral hypoxia but also by genetic alterations, such as gain-of-function mutations in oncogenes and loss-of-function mutations in tumor-suppressor genes.28) Moreover, growth factors and cytokines stimulate HIF-1α synthesis via the oxygen-independent activation of phosphatidylinositol 3-kinase (PI3K)29,30) or mitogen-activated protein kinase (MAPK) pathways.31) VHL mutation also leads to over expression of the HIF-1α protein.32) Therefore, lack of a routine, easy and dynamic method to monitor HIF-1 activity in solid tumors remains a major problem.

In the present study, we developed a suitable and convenient system to monitor HIF-1 activity in real-time in tumor xenografts by using the 5HRE promoter.21,22) Destabilized Enhanced GFP (d2EGFP) was chosen as the reporter gene because it can be detected without any substrate or cofactor, and especially because the half-life of d2EGFP is reduced to less than 2 hours. Such short half-life is due to the residues 422–461 of mouse ornithine decarboxylase protein (MODC domain) fused in d2EGFP protein, and makes it possible to observe HIF-1 activity in real-time.33) We were consequently able to demonstrate that d2EGFP fluorescence is observed in vitro only under hypoxic conditions and in vivo in the regions stained with the hypoxia marker.

MATERIALS AND METHODS

DNA constructs

The DNA fragments encoding EGFP and d2EGFP were inserted between the NcoI and NotI recognition sites of the pEF/myc/cyto vector (Invitrogen, Carlsbad, CA) to construct the plasmids pEF/EGFP and pEF/d2EGFP, respectively. In addition, the DNA fragment of the 5HRE-hCMVmp enhancer/promoter22) was inserted between the KpnI and NcoI sites of pEF/EGFP and pEF/d2EGFP to construct the plasmids p5HRE-EGFP and p5HRE-d2EGFP, respectively. To construct the plasmid, pCMV-d2EGFP, the DNA fragment of the 5HRE-hCMVmp enhancer/promoter of p5HRE-d2EGFP was substituted for that of the intact CMV promoter.22)

Cell culture and transfection

The human melanoma cell line Be1134) (the gift from Dr. Oya) was cultured in 20% FCS-MEM-1. To establish the stable transfectants, the cells were seeded on a 60mm culture dish and transfected with 5 µg of pCMV-d2EGFP, p5HRE-EGFP or p5HRE-d2EGFP by using the Superfect Transfection Reagent (QIAGEN, Valencia, CA), according to the manufacture’s instructions. Three hours after the transfection, the culture medium was changed to the medium containing 800 µg/ml of G418 disulfate (Nacalai Tesque, Kyoto, Japan), an antibiotic to select the stable transfectant, and the cells were cultured for an additional 2 weeks. With the aid of a fluorescent microscope with a 488 / 510 filter, a stable transfectant with strong fluorescence was isolated from the cells transfected with pCMV-d2EGFP plasmid. The stable transfectant with p5HRE-d2EGFP, on the other hand, was treated under hypoxic conditions (0.02%) for 20 hours before being isolated.

Hypoxia treatment and reoxygenation

Cells were treated under various hypoxic conditions. In one procedure, 90% N2, 5% H2 and 5% CO2 with a palladium catalyst were used in an Environment Chamber (Sheldon Manufacturing, Inc., Cornelius, OR). In another, the cells were treated in pre-warmed aluminum chambers by means of exhausting and gassing with 95% N2 and 5% CO2 for various cycles to produce oxygen concentrations of 2%, 0.2%, 0.02% and <0.01%. After the hypoxic treatment (0.02% O2) for 20 hours, the cells were cultured in the incubator with 95% air and 5% CO2 for the times indicated in each figures to reoxygenate the cells.

Flow cytometry analysis

Green fluorescence was analyzed with a flow cytometer (Becton Dickinson, San Jose, CA), and the data were processed with CellQuest software (Becton Dickinson). Histograms of GFP were plotted with the log scale for GFP fluo-
Fig. 1. Hypoxia-responsible d2EGFP expression in Be11/5HRE-d2EGFP stable transfectant. (A) Schematic diagrams of the CMV-d2EGFP gene (top) and the 5HRE-d2EGFP gene (bottom). (B) Both stable transfectants Be11/5HRE-d2EGFP (a, c) and Be11/CMV-d2EGFP (b, d) were treated under hypoxic condition (0.02% O₂) for 20 hours, and reoxygenated for 4 hours. (C) After the same treatment as described in (B), Be11/CMV-d2EGFP (a) and Be11/5HRE-d2EGFP (b) were subjected to FACS analysis and histograms of their fluorescent intensity are shown.
rescence and the results were recorded as means of the middle of the peaks.

**Immunohistochemical analysis**

Stable transfectants of Be11 with pCMV-d2EGFP or p5HRE-d2EGFP (10^6 cells in 100 µl of PBS) were subcutaneously (s.c.) injected into the flank or leg of 6–8 weeks nude mice (BALB/c nu/nu; CLEA Japan, Inc., Tokyo, Japan). When the tumor diameter exceeded 15 mm, the tumor bearing mice were intraperitoneally (i.p.) injected with pimonidazole hydrochloride (60mg/kg), and 90 min later, the solid tumors were surgically removed and fixed in 10% Formalin Neutral Buffer Solution pH 7.4 (Wako Pure Chemical Industries, Osaka, Japan). Immunostaining for the detection of d2EGFP expression and hypoxic cells was carried out with the anti-GFP Ab (BD Living Color™ A.v. Peptide Antibody; Clontech, Franklin Lakes, NJ) and with anti-pimonidazole Ab (Hypoxyprobe-1 kit; Natural Pharmacia International, Inc., Belmont, MA), respectively, by the indirect immunoperoxidase method as described previously. To calculate the percentage of pimonidazole-positive cells and d2EGFP-expressing cells in the Be11/5HRE-d2EGFP solid tumor, the corresponding pimonidazole-stained cells and d2EGFP-stained cells were quantified with NIH Image 1.63 software, and compared to the quantity of the whole tumor.

**Fluorescent microscope analysis and imaging of HIF-1 activity in vivo**

Microscopic analysis was conducted with an Olympus BX-60 microscope fitted with Olympus U-MWIB filter cubes (460–490 nm for excitation filter, 505–550 nm for dichroic filter and 515–550 for emission filter). Images were taken with a Sensys CCD Camera (Photometric, Livingston, UK). Blue color excitation light and yellow filters were used for the in vivo real-time imaging.

**RESULTS**

**Hypoxia-inducible fluorescence from cells transfected with p5HRE-d2EGFP**

To establish a convenient system to visualize the HIF-1 transcriptional activity in tumor xenografts in real-time, we first constructed the p5HRE-d2EGFP plasmid (Fig. 1A). A

![Fig. 2. Effects of various hypoxic treatments on d2EGFP fluorescence. (A) After hypoxic treatment (0.02% O_2) of various durations, fluorescent intensity of Be11/5HRE-d2EGFP stable transfectants was analyzed with FACS and is shown as a histogram. (B) Ratios of fluorescent intensity of the same cell preparations as in (A) to the intensity without hypoxic treatment are shown as relative fluorescence units. (C) After various hypoxic treatments (2%, 0.2%, 0.02% and <0.01%) for 20 hours, fluorescent intensity of Be11/5HRE-d2EGFP stable transfectants was analyzed and is shown as a histogram. (D) Ratios of fluorescent intensity of the cells after the same hypoxic treatments as in (C) to the intensity after aerobic treatment are shown as relative fluorescence units. (E) After hypoxic treatment (0.02% O_2) for 20 hours, Be11/5HRE-d2EGFP stable transfectants were reoxygenated for various durations and the fluorescent intensity was analyzed and is shown here as a histogram. (F) Ratios of fluorescent intensity of the cells after the same treatment as in (E) to the intensity after aerobic treatment are shown as relative fluorescence units. Be11/5HRE-EGFP stable transfectants (solid triangles), Be11/5HRE-d2EGFP stable transfectants (solid squares).**
5HRE promoter was employed to induce reporter gene expression under the control of HIF-1 activity,\textsuperscript{21,22} and d2EGFP was used as a reporter gene, because its half-life is reported to be somewhat less than 2 hours,\textsuperscript{33} which we thought should enable us to observe HIF-1 activity in real-time. The human melanoma cell line Be11 was stably transfected with the plasmid p5HRE-d2EGFP, and a clone was isolated by taking advantage of the hypoxia-dependency of the d2EGFP expression (Fig. 1A). In addition, Be11, this time stably transfected with pCMV-d2EGFP plasmid, was established as a control with constitutive expression of d2EGFP (Fig. 1A). To confirm the occurrence of hypoxic induction, both stable transfectants were processed in 0.02% of oxygen concentration. The fluorescence microscopic examination clearly showed that Be11/5HRE-d2EGFP transfectants expressed negligible levels of fluorescence under aerobic conditions but adequate levels of fluorescence after hypoxic treatment (Fig. 1Ba and c). On the other hand, Be11/CMV-d2EGFP transfectants showed bright green fluorescence under both aerobic and hypoxic conditions (Fig. 1Bb and d).

FACS analysis also clearly showed fluorescent intensity in both cell lines (Fig. 1C). In the cell population of Be11/5HRE-d2EGFP transfectants, a more than 200-fold induction of d2EGFP fluorescence was observed after hypoxic treatment compared to after aerobic treatment (Fig. 1Cb), whereas constitutive fluorescence was observed after both aerobic and hypoxic treatment in the Be11/CMV-d2EGFP transfectants (Fig. 1Ca). These results indicate that the 5HRE-CMVmp promoter can be used as a specific and robust hypoxia-inducible promoter. Although hypoxic treatment itself seemed to somewhat diminish the brightness of the Be11/CMV-d2EGFP transfectants (Fig. 1Ca), such difference was hardly detectable by fluorescence microscopic analysis (Fig. 1Bb and d).

**Effects of various hypoxic treatments on d2EGFP fluorescence**

In order to analyze the effects of hypoxic treatment of various durations on fluorescent intensity, Be11/5HRE-d2EGFP transfectants were treated under hypoxic conditions for 0, 2, 6, 12, and 24 hours, after which the green fluorescence was monitored by means of FACS analysis. Even after 2 hours of hypoxic treatment, fluorescence was detected with fluorescent microscopic as well as with FACS analysis (Fig. 2A and data not shown). Increases in both fluorescent intensity and number of fluorescent cells depended on the duration of hypoxic treatment up to 24 hours, after which saturation levels were reached (Fig. 2A and B).

To assess the oxygen concentration required for inducing d2EGFP expression, Be11/5HRE-d2EGFP cells were treated for 20 hours with various oxygen concentrations, that is, <0.01%, 0.02%, 0.2% and 2%. FACS analysis showed that d2EGFP expression increased when the oxygen concentra-

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Fig. 3. Real-time imaging of HIF-1 activity in the Be11/5HRE-d2EGFP tumor xenograft. (A), (B) Heterogeneous fluorescence in the tumor xenograft with Be11/5HRE-d2EGFP cells was detected by means of blue excitation light and a yellow filter. (C) Homogeneous fluorescence in the tumor xenograft with Be11/CMV-d2EGFP cells was used as control.
tion was reduced from 20% to 0.2%, and maintained a high expression level from 0.2% to anaerobiosis (<0.01%) (Fig. 2C and D). Even at the 2% oxygen concentration, fluorescent intensity increased almost 1,000 times compared to the one at 20% oxygen concentration (Fig. 2D), and could be detected directly with the fluorescence microscope (data not shown).

Next we analyzed the effect of reoxygenation on d2EGFP fluorescence, because oxygen molecules are reported to be necessary for chromophore formation of GFP and its derivatives. Be11/5HRE-d2EGFP cells were treated under hypoxic conditions (0.02% O₂) for 20 hours, and reoxygenated for various lengths of time in the incubator with 95% air and 5% CO₂. The hypoxic treatment even without any reoxygenation enhanced fluorescence by a factor of more than 200 compared to that observed under aerobic conditions (Fig. 2E and F). Reoxygenation for 4 hours resulted in a sharp and high peak of fluorescence, after which the fluorescence levels decreased dramatically. To examine the effect of the MODC domain fused in d2EGFP on protein stability, we established the Be11/5HRE-EGFP cells, in which expression of the EGFP protein (without MODC domain) was controlled by the 5HRE promoter. When the Be11/5HRE-EGFP cells were subjected to the same experiment as described above, they showed more stable fluorescence than the Be11/5HRE-d2EGFP cells during the observation period (Fig. 2F). These results clearly demonstrate that the decrease in the fluorescence of Be11/5HRE-d2EGFP was due to the MODC domain of the d2EGFP protein.

**Real-time imaging of HIF-1 transcriptional activity in tumor xenograft**

In order to visualize the HIF-1 transcriptional activity in a solid tumor in a living mouse, Be11/5HRE-d2EGFP cells were s.c. inoculated into nude mice. One month after the implantation, these cells formed solid tumors with diameter of more than 15 mm. Real-time imaging under the blue exci-
Characterization of GFP-expressing cells by immuno-histochemical analysis

HIF-1 activation is induced by not only intratumoral hypoxia but also the other stimuli. To determine, therefore, whether hypoxic stimuli definitely up-regulated the 5HRE-promoter activity in our in vivo study, the regions expressing d2EGFP in the solid tumor were compared to those stained with the hypoxia marker pimonidazole. In the Be11/CVM-d2EGFP solid tumor, homogeneous and strong expressions of d2EGFP were detected with the anti-GFP Ab, which recognizes all of GFP derivatives including d2EGFP (Fig. 4A). HE-stained section of Be11/CVM-d2EGFP xenograft revealed that the d2EGFP-expressing cells were in the viable regions (Fig. 4B). On the other hand, heterogeneous expressions of d2EGFP were observed in the tumor xenografts with Be11/5HRE-d2EGFP cells (Fig. 4C). The d2EGFP-positive cells were located in the boundary area between the well-oxygenated viable regions and the necrotic regions (Fig. 4C and D). Similar regions in the Be11/5HRE-d2EGFP tumor also stained with antipimonidazole Ab, (Fig. 4E).

Higher magnifications of the square areas in Fig. 4C, 4D and 4E are shown as Fig. 4Fa, 4Ga and 4Ha respectively. In the HE-stained specimen, red blood cells clearly showed the location of tumor blood vessels (Fig. 4G), which were surrounded by viable tumor cells in direct proximity, and moreover by necrotic tumor cells at a greater distance. To be precise, the necrotic regions were located more than 100 µm from the blood vessels. In addition to the HE-stained specimens, the higher-magnified images stained with anti-GFP Ab clearly demonstrated that the d2EGFP-expressing cells were located about 100 µm from the tumor blood vessels (Fig. 4F). Finally, the staining pattern obtained with anti-GFP Ab was very similar to that produced by the hypoxia marker pimonidazole (Fig. 4F and H). These results strongly suggest that the 5HRE-system induces d2EGFP expressions in hypoxic tumor cells.

In the specimens stained with anti-GFP Ab and antipimonidazole Ab, it was also clear that more cells were stained with the former than with the latter. For quantification, both specimens were analyzed with NIH Image 1.63 software. The percentage of pimonidazole-positive cells in the whole solid tumor was 9.2 ± 3.8% (n = 5), while that of d2EGFP-expressing cells in the whole solid tumor was significantly higher, 22.7 ± 6.1% (n = 5; p < 0.01).

DISCUSSION

HIF-1 induces various kinds of hypoxia-responsive gene expressions, which are related to the malignant phenotype of tumor cells, such as angiogenesis, metastasis, invasion and evasion of apoptosis. The lack of a technique that allows for convenient imaging of HIF-1 transcriptional activity therefore remains as one of the problems for basic research aiming at the development of anticancer drugs in cancer treatment. In order to address this issue, we developed an imaging system using both an HIF-1-responsive promoter and a specific reporter protein. Shibata et al. examined various combinations of HRE and promoters to achieve higher responsiveness of promoter activity to hypoxic stress, and demonstrated that the combination of 5HRE and a CMVmp induces 500 fold more gene expression under hypoxic conditions than under aerobic conditions. Moreover, the expression level under hypoxic conditions is almost same as the one by the intact CMV promoter. We therefore employed the 5HRE-hCMVmp enhancer/promoter system for the study presented here. In agreement with previously reported results, only negligible levels of reporter expression were observed under aerobic conditions but these levels became clearly detectable after hypoxic treatment. For this reason, the 5HRE enhancer/promoter met our requirements better than any other regulatory systems.

We chose a derivative of GFP protein as the reporter gene because no additional cofactor or substrate is needed for its visualization. Attention must be paid, however, to applying GFP derivatives to in vivo imaging because anoxic conditions inhibit chromophore formation. In fact, we observed in the FACS analyses that hypoxic treatment itself seemed to diminish the brightness of the Be11/CVM-d2EGFP transfectants somewhat (Fig. 1Ca), and that d2EGFP fluorescence increased after reoxygenation (Fig. 2E and F). But when we treated the cells with the translation inhibitor, cyclohexamide, just after the hypoxic treatment, the d2EGFP signal did not increase after reoxygenation (data not shown). This finding suggests that the increase in fluorescence after reoxygenation was not mainly the result of chromophore formation but of translation. We can therefore conclude that chromophore formation was not significantly inhibited in our experimental setting. Additionally, such small differences in fluorescent intensity were hardly detectable with fluorescence microscopic analysis (Fig. 1Bb and d). Thus, there seemed to be no serious obstacles to employing d2EGFP as a reporter for our purpose.

The 5HRE enhancer/promoter system has been used to analyze the effect of x-ray irradiation on tumor reoxygenation as well as on subsequent HIF-1 activation. The HIF-1-mediated promoter with eight copies of HRE has also been used to develop a novel PET imaging system. In both cases, however, assessment in real-time seemed to be difficult,
because the reporter proteins were so stable\textsuperscript{33} that some of them may have remained in the cells after reoxygenation. The reason why we choose d2EGFP rather than the other GFP derivatives was the short half-life of the protein, which is caused by the MODC domain fused in it.\textsuperscript{33} In fact, we were able to demonstrate that d2EGFP fluorescence has a more rapid turnover than that of EGFP (Fig. 2F). Moreover, when we treated the cells with cyclohexamide just after the hypoxic treatment and just before reoxygenation, the d2EGFP fluorescence disappeared within 2 hours (data not shown). This MODC activity enabled us to assess HIF-1 activity in solid tumors in real-time.

Be11/CMV-d2EGFP showed homogeneous fluorescence in the whole tumor, while heterogeneous occurrence of green fluorescence was directly visualized in tumor xenografts inoculated with Be11/5HRE-d2EGFP cells. Our in vitro study enabled us to estimate to some extent the hypoxic status in the solid tumor. As Be11/5HRE-d2EGFP expressed detectable amounts of fluorescence in response to different hypoxic stimuli, e.g. 0.02% O\textsubscript{2} for 2 hours and <2% O\textsubscript{2} for 20 hours, we can assume that the fluorescent cells in the solid tumors were exposed to at least these conditions. At this time, however, an accurate assessment of tumor hypoxia with this method alone is not possible. For instance, it was difficult to distinguish the acute hypoxia from the chronic one. Moreover, we might not have visualized the acute hypoxia, but chronic one alone, because most gene expressions take a certain time. Therefore, combining our method with computer based image analysis and/or flow cytometry analysis can be expected to expand the efficacy and application of 5HRE-d2EGFP as an imaging system.

Immunohistochemical analysis clearly showed that the d2EGFP-expressing cells are located at a distance of about 100 \mu m from the tumor blood vessels, and the staining pattern is almost the same as the one obtained with anti-pimonidazole Ab. These results suggest that the 5HRE enhancer/promoter induces d2EGFP expressions in response to hypoxic stimuli in the solid tumor. However, more highly magnified images showed that more cells are stained with anti-GFP Ab than with anti-pimonidazole Ab in the boundary area between viable and necrotic regions (Fig. 4F and H). The quantitative analysis using NIH Image 1.63 software also clearly showed that there were certainly more GFP-expressing cells than pimonidazole-positive cells. Although these results might have been caused simply by the different sensitivities of the Abs, it is also possible that the HIF-1 activity is up-regulated by another regulatory pathway independent of hypoxia. The up-regulation of HIF-1\textalpha activity has been observed even under normoxic conditions in renal carcinoma cells, in which the von Hippel-Lindau tumor suppressor gene had lost its function.\textsuperscript{35,36} Furthermore, HIF-1 activity increases when the PI3K/AKT signaling pathway is activated.\textsuperscript{30} Regardless of the HIF-1-activating mechanisms, the 5HRE-d2EGFP system can reflect the HIF-1 activity, so that this system can be used for monitoring HIF-1 activity in real-time in vivo.

It has been reported that HIF-2 as well as HIF-1 regulates gene expression of VEGF, glycolytic enzymes and other HRE-driven genes in response to hypoxia.\textsuperscript{39} Although HIF-2 plays some important roles in physiological stress response, the details of its mechanism and how it differs from that of the HIF-1 pathway remain unknown. To examine whether the 5HRE enhancer/promoter system is regulated by HIF-2 activity, too, we assessed its hypoxia responsiveness by using a squamous cell carcinoma cell line, in which the HIF-1\textalpha gene was mutated, and found evidence of the HIF-2-dependency of the 5HRE system. (in preparation). These findings indicate that this monitoring method can be used to analyze details of the activities of the HIF family in vivo. Moreover, this method can be expected to contribute to the development of HIFs-targeting drugs,\textsuperscript{28} such as YC-1\textsuperscript{40} and TOP3.\textsuperscript{5,35,41}

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