J. Radiat. Res., 52, 545–556 (2011)

Award Article

How Can We Overcome Tumor Hypoxia in Radiation Therapy?

Hiroshi HARADA*

Tumor microenvironment/Hypoxia/Hypoxia-inducible factor 1 (HIF-1)/Radioresistance/Hypoxia Image-Guided Radiation Therapy (Hypo-IGRT).

Local recurrence and distant metastasis frequently occur after radiation therapy for cancer and can be fatal. Evidence obtained from radiochemical and radiobiological studies has revealed these problems to be caused, at least in part, by a tumor-specific microenvironment, hypoxia. Moreover, a transcription factor, hypoxia-inducible factor 1 (HIF-1), was identified as pivotal to hypoxia-mediated radioresistance. To overcome the problems, radiation oncologists have recently obtained powerful tools, such as "simultaneous integrated boost intensity-modulated radiation therapy (SIB-IMRT), which enables a booster dose of radiation to be delivered to small target fractions in a malignant tumor", "hypoxia-selective cytotoxins/drugs", and "HIF-1 inhibitors" *etc*. In order to fully exploit these innovative and interdisciplinary strategies in cancer therapy, it is critical to unveil the characteristics, intratumoral localization, and dynamics of hypoxia/HIF-1-active tumor cells during tumor growth and after radiation therapy. We have performed optical imaging experiments using tumor-bearing mice and revealed that the locations of HIF-1-active tumor cells changes dramatically as tumors grow. Moreover, HIF-1 activity changes markedly after radiation therapy. This review overviews 1) fundamental problems surrounding tumor hypoxia in current radiation therapy, 2) the function of HIF-1 in tumor radioresistance, 3) the dynamics of hypoxic tumor cells during tumor growth and after radiation therapy, and 4) how we should overcome the difficulties with radiation therapy using innovative interdisciplinary technologies.

INTRODUCTION

The discovery of X-rays in 1895 by Wilhelm Conrad Röntgen opened the door for radiation therapy, one of the three major therapeutic treatments for cancer.[1] Radiation therapy has developed and improved through the integration of technologies and knowledge of engineering, physics, biology, and chemistry.[2] Also, collaborations between scientists and clinicians have greatly contributed to the development; namely, basic scientists have proposed novel concepts in radiation therapy, and radiation oncologists, on the other hand, have examined their therapeutic effects and guided improvements.[2] However, even the most innovative strategy has failed to achieve a complete remission, and patients often suffer from tumor recurrence and/or distant metastasis after radiation therapy. To overcome these problems, it is critical to elucidate mechanisms by which cancer cells survive, recur, and metastasize after radiation therapy.

The presence or absence of molecular oxygen is known to influence the biological effect of ionizing radiation; cells obtain radioresistance under hypoxic conditions.[3] This phenomenon, known as the "oxygen effect", has drawn considerable attention in radiation oncology ever since Thomlinson and Gray reported that malignant tumors are composed of hypoxic as well as well-oxygenated cancer cells.[4] Radiation chemical studies have elucidated that the depletion of oxygen results in the inefficient formation of DNA strand breaks by ionizing radiation, and moreover, prevents the damage from being repaired.[3] Meanwhile, radiation biological studies have revealed that hypoxic stimuli trigger changes in both the "DNA damage repair pathway"[5] and "cell death/survival signaling pathway", leading to an increase in the cellular radioresistant phenotype. Moreover, it was proved that a transcription factor, hypoxia-inducible factor 1 (HIF-1), plays a pivotal role in hypoxia-related tumor radioresistance.[6–9]

To overcome the problems of tumor hypoxia, several therapeutic strategies have been developed. Treatment under hyperbaric oxygen conditions aims to deliver radiation to

*Corresponding author: Phone: +81-75-753-9301,
Fax: +81-75-753-9281,
E-mail: hharada@kuhp.kyoto-u.ac.jp
Group of Radiation and Tumor Biology, Career-Path Promotion Unit for Young Life Scientists, Kyoto University, Yoshida Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan.

All of the experiments with animals were approved by the Animal Research Committee of Kyoto University.

doi:10.1269/jrr.11056

well-oxygenated and less-hypoxic tumor tissues.[10] Fractionated radiation therapy targets hypoxic tumor cells that have just been reoxygenated as a result of ex-irradiation.[11–13] In addition, radiation oncologists have recently obtained two powerful new tools; "simultaneous integrated boost intensity-modulated radiation therapy (SIB-IMRT)" which enables the delivery of a booster dose of radiation, especially to small fractions of cells in a malignant tumor,[14] and "hypoxia-selective cytotoxins/drugs".[3] To exploit these innovative and interdisciplinary strategies in radiation therapy, it is critical to unveil the characteristics, distribution, and dynamics of hypoxic tumor cells during tumor growth and after radiation therapy.

I and my colleagues have performed optical imaging experiments using tumor-bearing mice to detect tumor hypoxia/HIF-1 activity and revealed that the location of hypoxic tumor cells/HIF-1 activity changes dramatically as the tumor grows.[15,16] Moreover, the change in the volume of hypoxic regions is also dramatic after radiation therapy.[17,18] Here, I review the fundamental problem of tumor hypoxia in current radiation therapy, dynamics of hypoxic tumor cells during tumor growth and after radiation therapy, and how we can overcome the difficulties with tumor hypoxia in radiation therapy using innovative technologies or hypoxia-targeted drugs.

A TUMOR-SPECIFIC MICROENVIRONMENT, HYPOXIA

Aberrantly accelerated proliferation caused by the activation of oncogenes and/or disruption of tumor suppressor genes is a typical feature of cancer cells[19] and leads to an imbalance between oxygen supply to and oxygen consumption in a malignant solid tumor. Such disequilibrium as well as inadequate diffusion of molecular oxygen within a tumor is a major cause of the highly heterogeneous and severely compromised oxygenation of tumors (Fig. 1).[4,20] Tumor cells proliferate and grow actively only if supplied with oxygen and nutrients from tumor blood vessels (normoxic regions),[21] and inevitably die in areas about 100 μm from tumor blood vessels (necrotic regions).[21] Between these two regions, there exist so-called chronic hypoxic areas, in which cancer cells obtain the minimal levels of oxygen necessary for their survival.[21]

In addition to chronic hypoxia, acute/intermittent/cycling hypoxia has received much attention because of its relevance to malignancy and radioresistance. Acute hypoxia was first recognized by Brown *et al*. in 1979,[22] who mentioned that a malformed tumor vasculature causes the transient opening and closing of blood vessels, changes in blood flow, fluctuations of tumor perfusion, and ultimately transient hypoxia even within 70 μm tumor blood vessels. Subsequent studies showed that at least 20% of cancer cells experience acute hypoxia in malignant solid tumors, supporting the notion that acute as well as chronic hypoxia is a common feature of solid tumors.[23,24] Moreover, it is generally recognized that hypoxia is unique to malignant solid tumors; and therefore, can be used to distinguish cancer from normal cells and to develop anticancer strategies with high tumor-specificity.[3]

RADIOCHEMICAL AND RADIOBIOLOGICAL MECHANISMS BEHIND TUMOR RADIORESISTANCE UNDER HYPOXIA

Oxygen effect: Radioresistance of cancer cells under hypoxic conditions

The presence or absence of molecular oxygen influences the biological effect of x-rays.[3,4] This phenomenon, the so-called oxygen effect, was first recognized in 1912 with the

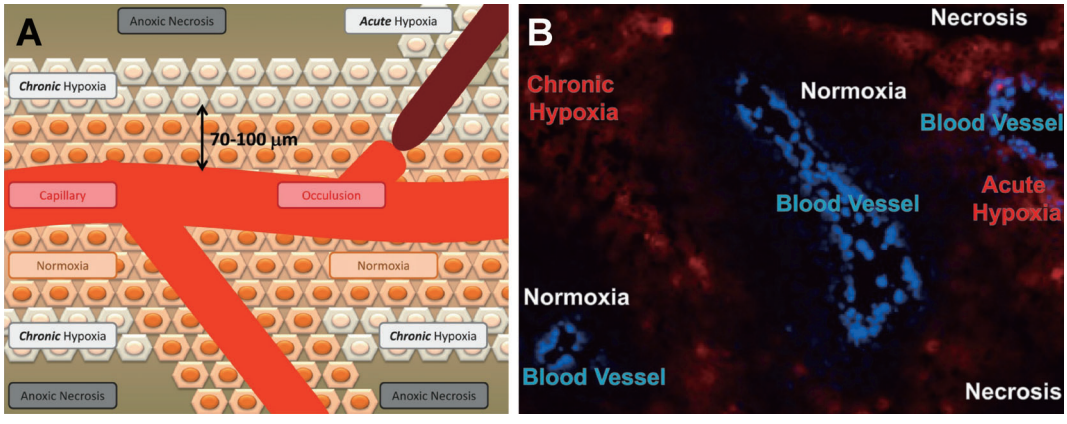

Fig. 1. Spatial relationship between blood vessels and hypoxia in a malignant solid tumor. Chronic hypoxia exists 70–100 μm from tumor blood vessels. Acute/cycling hypoxia caused by fluctuations in tumor blood flow occurs proximal to tumor blood vessels. **A)** Schematic diagram of tumor microenvironments. **B)** Frozen section of a tumor xenograft was stained with anti-HIF-1α antibody (red fluorescence). Tumor blood vessels can be seen as blue fluorescence (Hoechst33342 perfusion marker).

observation that the reaction to a radium applicator decreased when it was pushed tight onto the skin and the blood flow there decreased. The oxygen effect was further confirmed by Gray *et al*. through a quantitative analysis of the oxygen effect on growth inhibition of the primary root of a broad bean.[25] A milestone study linking the oxygen effect with radioresistance of cancer cells was published by Thomlinson and Gray in 1955.[4] They proposed that oxygen levels decreased in a solid tumor through successive layers of cancer cells distal to blood vessels and cancer cells with a distance of about 10 cell diameters from the vessels are viable but radioresistant. Although there is no direct *in vivo* evidence that this population survives radiation therapy better than well-oxygenated cancer cells and causes local tumor recurrence, this idea is generally supported by extensive studies *in vitro*, such as clonogenic assays *etc*.[3] Thus, hypoxic tumor cells have been recognized as a major obstacle to radiation therapy.[3,26]

Radiation chemical mechanisms behind the oxygen effect

Although the mechanism of the oxygen effect is still not fully understood, it is generally assumed that oxygen acts at the level of free radicals.[3,21] Ionizing radiation induces ionization in or close to the genomic DNA of target cells and produces various radicals, which cause DNA strand breaks. Oxygen oxidizes the DNA radicals and is known to make the damage permanent. On the other hand, in the absence of oxygen, the DNA radicals are reduced by compounds containing sulfhydryl groups (SH groups), which restore/repair the DNA to its original form. Taken together, DNA damage, especially irreparable double stranded breaks, is significantly less severe in the absence of molecular oxygen, leading to hypoxia-related radioresistance of cancer cells.

Radiation biological mechanisms behind the oxygen effect

In addition to radiochemical mechanisms, radiobiological mechanisms are also important. It has been elucidated that hypoxic stimuli trigger changes in both the "DNA damage repair pathway"[5] and the "cell death/survival signaling pathway", leading to an increase in the radioresistant phenotype. Moreover, accumulated evidence from molecular biology and radiation oncology revealed an important role of a transcription factor, hypoxia-inducible factor 1 (HIF-1).[6–9] Expression of the alpha subunit of the HIF-1 (HIF-1α) protein as well as the intratumor hypoxic fraction has been reported to correlate with a poor prognosis, local tumor recurrence, and distant tumor metastases after radiation therapy.[27–29] Inhibition of intratumor HIF-1 activity by a pharmacological HIF-1 inhibitor, YC-1, a dominant negative HIF-1α, and knockdown of HIF-1α expression with short hairpin RNA or short interfering RNA, respectively, delayed tumor growth after radiation.[7–9,18,30] In the following, I especially focus on recent advances in our understanding of how HIF-1 induces tumor radioresistance.

Regulation of HIF-1 activity

HIF-1 is a heterodimeric transcription factor composed of an α-subunit (HIF-1α) and a β-subunit (HIF-1 β/ARNT).[31] Its hypoxia-dependent activity is regulated at various levels, such as translational initiation and posttranslational modifications and so on.[32,33]

The best characterized mechanism is that influencing the stability of HIF-1α. Under normoxic conditions, the oxygen-dependent degradation (ODD) domain of HIF-1α is hydroxylated by prolyl hydroxylases and then ubiquitinated by a pVHL-containing E3 ubiquitin ligase, resulting in rapid degradation of the HIF-1α protein.[32–35] On the other hand, HIF-1α is stabilized and activated under hypoxic conditions and interacts with its binding partner, HIF-1β.[31] The resultant HIF-1 binds to its cognate transcriptional enhancer sequence, the hypoxia-responsive element (HRE), and induces the expression of various genes related to angiogenesis, glycolysis, and the invasion and metastasis of cancer cells.[36–40] In addition to the PHDs-VHL-dependent mechanism, HIF-1α is known to be degraded in a receptor of activated protein kinase C (RACK1, also known as GNB2L1)-dependent manner.[41] RACK1 binds to HIF-1α in competition with heat shock protein 90 which stabilizes the HIF-1α protein. Therefore, interaction with RACK1 leads to the oxygen-independent degradation of HIF-1α.

It was recently elucidated that production of the HIF-1α protein is dependent on a phosphatidylinositol 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) signaling transduction pathway.[42,43] The 5'-untranslated region of HIF-1α mRNA contains a 5'-terminal oligopolypyrimidine tract which regulates the translational initiation of HIF-1α in response to the activation of mTOR and its downstream factor, p70 S6 kinase and eukaryotic initiation factor-4E (eIF-4E).[43] Therefore, the Akt-mTOR signaling pathway stimulates the translation of HIF-1α even under normoxic conditions in response to certain growth factors, cytokines, and signaling molecules, such as epidermal growth factor (EGF), fibroblast growth factor 2 (FGF-2), heregulin, insulin, insulin-like growth factor (IGF)-1 and IGF-2, and interleukin 1β (IL2β).[43–47]

The post-translational modification of HIF-1α also plays an important role in stimulating 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-1α.[32,33,48] The hydroxylation blocks the interaction of HIF-1α with the transcriptional co-factors p300 and CBP, resulting in the suppression of HIF-1's transactivational activity. Because oxygen is a substrate for the hydroxylation of asparagine by FIH-1, HIF-1's transactivational activity is restored under oxygen-deprived hypoxic conditions. In addition, the transactivational activity of HIF-1 is stimulated through the phosphorylation of HIF-1α by

signaling *via* ERK (p42 and p44) and p38 MAP kinase pathways.[49,50]

Function of HIF-1 in radioresistance of hypoxic tumor cells

An attractive model of the role of HIF-1 in cellular radioresistance under hypoxia was proposed recently; 1) radiation activates HIF-1 in a solid tumor, 2) HIF-1 induces the expression of VEGF, 3) VEGF protects endothelial cells from the cytotoxic effects of radiation, and 4) the radioprotected tumor blood vessels assure the supply of oxygen and nutrients to tumor cells and promote tumor growth.[7,18,51,52] This model is supported by the following results. Optical real-time imaging experiments using a HIF-1-dependent reporter gene revealed that intratumor HIF-1 activity is dramatically induced by radiation therapy, supporting the first step of the model.[7,17,18,53] Hypoxia-conditioned medium containing a high level of VEGF significantly decreased the incidence of radiation-induced apoptosis of human umbilical vein endothelial cells (HUVECs) *in vitro*.[18,51,52] A HIF-1 inhibitor, YC-1, or a neutralizing antibody against VEGF dramatically induced apoptosis of endothelial cells and decreased micro vessel density after radiation therapy, resulting in a radiosensitizing effect in a tumor growth delay assay.[7,18,54]

INTRATUMORAL DYNAMICS OF HYPOXIC AND HIF-1 ACTIVE CELLS

As mentioned above, the radioresistance of cancer cells is influenced by both oxygen concentrations and HIF-1 activity; therefore, it is important to improve our basic understanding of the intratumor localization and dynamics of them for the development of innovative therapeutic strategies.

Spatio-temporal dynamics of hypoxic areas

Because chronic hypoxia basically exists 70–100 μm from tumor blood vessels, it is easy to predict its intratumoral distribution and volume histologically.[3,4,21,26] On the other hand, acute/cycling hypoxia is more complicated because it is influenced by various factors: erythrocyte flux, neoangiogenesis, transient opening and closing of blood vessels (vessel occlusion), changes in blood flow, and fluctuations of tumor perfusion.[22,24] A double labeling method using two kinds of hypoxia tracers revealed that acute/cycling hypoxia is ubiquitous in human tumors as well as tumor xenografts in experimental animals[55] and that 8 to 20% of tumor cells experience acute/cycling hypoxia.[56] Cycle frequency displays a diverse range from one cycle per minute (a dominant cycle time of 20–30 min)[57] to one cycle every several hours,[56] and moreover to one cycle per day (24 hours).[22] There is a clinical report that PET imaging for tumor hypoxia by using ^{18}F-misonidazole (^{18}F-MISO) in several patients at 3-days intervals showed changes in the position, shape, size, and intensity of the signal.[58,59]

Dynamics of hypoxic areas during and after radiation therapy

The oxygen-dependent difference in cellular radiosensitivity is associated with postirradiation alterations of the tumor microenvironment.[21] Namely, it has been assumed that the distribution of oxygen from tumor blood vessels to hypoxic tumor cells is dramatically improved after radiotherapy as a result of the death of well-oxygenated tumor cells and a subsequent decrease in oxygen consumption there.[21,60,61] This phenomenon is known as tumor reoxygenation. I and my colleagues actually confirmed the phenomenon in immunohistochemical analyses using tumor xenografts: hypoxic cells, which were originally detected with a hypoxia marker, pimonidazole, in the border between normoxic and necrotic regions, were not stained 6–24 hours after 5 Gy of X-irradiation.[17,18]

Dynamics of HIF-1-active cells/HIF-1 activity

To analyze the dynamics of HIF-1-active cells/HIF-1 activity in a malignant solid tumor, the best method is molecular imaging using HIF-1-dependent reporter genes. I and my colleagues developed a series of reporter plasmids, which express fluorescent proteins, *e.g.* enhanced green fluorescent protein (EGFP) and DsRed2, or bioluminescent proteins, *e.g.* firefly luciferase.[15,62–64] Cancer cells were stably transfected with the reporter gene and transplanted into immunedeficient nude mice. The resultant tumor-bearing mice with the reporter gene in their xenograft were subjected to real-time optical imaging experiments.

Dynamics of HIF-1-active tumor cells during tumor growth

Bioluminescent imaging, which is suitable for a quantitative analysis, revealed that the amount of intratumoral HIF-1 activity gradually increased as the xenograft grew.[15] On the other hand, fluorescent imaging, suitable for a spatiotemporal analysis at the micro-level, showed that the distribution of HIF-1-active cancer cells dramatically changed from day to day under the influence of neovascularization.[16] HIF-1-active tumor cells occurred far from tumor blood vessels. There is a strong correlation between the distance from HIF-1-active regions to the nearest blood vessel and the diameter of the vessel in the tumor xenograft model: Distance = $1.38 \times$ diameter + 19.4 ($r = 0.801$, $n = 25$).

Changes of HIF-1-activity after radiation therapy

The bioluminescent imaging using the tumor xenograft with the HIF-1-dependent promoter demonstrated that the level of intratumor HIF-1 activity decreased significantly and reached a minimum at 6 hours after 5 Gy of local ionizing radiation (early phase).[18] After that, HIF-1 activity increased and reached a plateau at 18–24 hours postirradiation (late phase),[17,18] although the timing and duration of the activation seems to depend on both the dose of radiation and the cell line.[7,18,53] Immunohistochemical analysis using HIF-1α antibody confirmed that the two phases of intratumor HIF-1 activity are dependent on the decrease and increase in

HIF-1α protein at 6 and 24 hours after radiation, respectively, in the border between normoxic/viable regions and necrotic regions.[17,18] Radiation-induced changes in the tumor microenvironment, especially in both glucose- and oxygen-availability (reoxygenation) in the border regions, plays an important role in the down-regulation and subsequent up-regulation of HIF-1α expression in both phases.[6,17,18] Immunostaining using a hypoxia marker, pimonidazole, revealed that the border regions were well-oxygenated (reoxygenated) in the early phase. PHD(s)-VHL-dependent degradation of HIF-1α dominates over the neo-synthesis of HIF-1α in the reoxygenated regions, resulting in low HIF-1 activity.[6,18] On the other hand, in the late phase, not only reoxygenation but also increased glucose-availability in the border regions dramatically up-regulates Akt-mTOR-dependent translation of HIF-1α.[6,17] Moreover, reoxygenation induces ROS production, which induces stabilization of the HIF-1α protein through the suppression of PHDs' activity.[7] In addition, reoxygenation increases NO levels in tumor-associated macrophages, too, leading to S-nitrosylation and the resultant stabilization of HIF-1α.[53] Overall, the neo-synthesis of HIF-1α exceeded its degradation in the late phase, resulting in the up-regulation of HIF-1 activity even under reoxygenated conditions.[6]

STRATEGIES TO ASSESS TUMOR HYPOXIA IN HUMAN CANCERS

Strategies to quantify and image hypoxia in clinical tumors have received considerable attention because of the significant impact of hypoxia/HIF-1 activity on the effect of radiation therapy. This section focuses on the development of strategies to assess tumor hypoxia in human cancers.

Direct measurement of partial oxygen pressure in solid tumors using a polarographic O_2 microelectrode

The polarographic needle electrode, first described by Weiss and Fleckenstein in 1986, avoided artifacts caused by compression and allowed direct and accurate measurements of O_2 tension in tumors.[65] Applying this system, Vaupel *et al*. clearly demonstrated the existence of a hypoxic microenvironment in solid tumors.[66] Median pO_2 values in normal breast and breast cancer are about 65 mmHg and 30 mmHg, respectively. Although this method had provided valuable information, it cannot be broadly applied in the clinic owing to its invasive nature and limitation to superficial tumors.

Immunohistochemistry

Tumor hypoxia confirmed with the polarographic needle electrode has been supported by immunohistochemical analyses using markers of hypoxia, such as pimonidazole[67] and EF5,[68] or using antibodies against hypoxia-related proteins, such as HIF-1α[69] and carbonic anhydrase IX.[70] These methods have been used to demonstrate the correlation between the extent of tumor hypoxia and prognosis of cancer patients after radiation therapy. However the immunohistochemical approach has weak points; it is highly invasive and not well-suited to a real-time analysis of tumor hypoxia.

Imaging of tumor hypoxia

New types of strategies referred to as functional/molecular imaging have been developed over the past two decades. They have been used to obtain information about the extent, location, and dynamics of tumor hypoxia with less invasiveness.

Positron emission tomography (PET) for tumor hypoxia

Increased glycolysis is a characteristic of malignant tumors.[71] It can be imaged using PET with a fluorinated non-metabolizable glucose analog, ^{18}F-fluorodeoxyglucose (^{18}F-FDG), as a tracer.[72] Because HIF-1 is responsible for both metabolism reprogramming from oxidative phosphorylation to glycolysis/lactic acid fermentation[73] and glucose uptake,[74] there is a possibility that ^{18}F-FDG accumulates in hypoxic regions. However, because many tumors are known to display increased rates of aerobic glycolysis (known as the "Warburg effect"[75]), there has been another claim that it is not a selective marker for tumor hypoxia.

Some nitroimidazole derivatives, such as ^{18}F-MISO, etanidazole pentafluoride (EF5), and ^{18}F-FPR170, and Cu(II)-diacetyl-bis(N4-methylthiosemicarbazone) (Cu-ATSM), have also been developed that selectively accumulate in hypoxic regions of solid tumors.[76–78] These compounds are known to be reduced under hypoxic conditions specifically, form a covalent bond with thiol groups of arbitrary proteins in the cell, and finally be detected by PET scanner. These approaches are now under investigation in clinical studies to examine their potential to predict treatment response, but will take time to be approved for routine use.

Through sequential hypoxia imaging using ^{18}F-MISO-PET with 3-days interval, Nehmeh *et al*. observed dynamic changes of the intensity distribution in head and neck cancers.[59] Comparative analysis of the sequential two datasets suggested that this variation was quite random, and therefore, leads to the hypothesis that the difference in spatial distribution was due to acute/intermittent/cycling hypoxia. Wang *et al*. successfully demonstrated the validity to discriminate acute from chronic hypoxia in serial PET images of cancer patients.[79]

Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI)

DCE-MRI is a noninvasive method of evaluating regional tumor blood flow as the temporal distribution of a small molecule tracer, D_2O, after its injection.[80] Information obtained by DCE-MRI enables us to predict the location of hypoxic regions in a solid tumor. Actually, a few studies have exploited this strategy to define a biological target volume to which an increased radiation dose is needed for

tumor eradiation.

Possible imaging strategies for tumor hypoxia: hopeful preclinical studies

Some hopeful strategies have been proposed and their possibilities were confirmed in preclinical studies.

Magat *et al*. demonstrated the usefulness of MR ^{19}F relaxometry map to analyze the spontaneous fluctuations of pO_2 over time in experimental tumor xenografts.[81] They generated pO_2 maps after direct intratumoral administration of a fluorine compound, hexafluorobenzene, whose relaxation rate (1/T1) is proportional to the partial oxygen pressure. They used a SNAP inversion-recovery sequence at 4.7 T to acquire parametric images of the T1 relaxation time with a high spatial and temporal resolution.

Dynamic three-dimensional electron paramagnetic resonance imaging (EPRI) has also been developed recently.[82,83] EPRI is a low-field magnetic resonance technique accomplishing quantitative and three-dimensional estimation of tissue oxygen concentration every 2–3 minutes with 1–2-mm spatial resolution. The collisional interaction of an exogenously administrated paramagnetic tracer with molecular oxygen makes the EPR line width broadened, and thus enables us to accomplish highly quantitative visualization of tissue oxygen concentration in real-time.

STRATEGIES TO OVERCOME RADIORESISTANCE OF HYPOXIC TUMOR CELLS

Based on the importance of hypoxic/HIF-1-active tumor cells in tumor radioresistance, strategies targeting them should have the potential to overcome the difficulties. This section focuses on progress in the development of hypoxia-targeted strategies in the field of radiation oncology (Table 1).

Increase in oxygen delivery

First, radiation oncologists tried to directly deliver more oxygen to locally advanced solid tumors during radiation. Hyperbaric oxygenation,[10] red blood cell transfusions,[84] and erythropoietin administration[85] were tried as methods to increase tumor oxygenation. Normobaric oxygen or carbogen has also been used and combined with nicotinamide, which is known to offset acute hypoxia.[86] Although these approaches showed therapeutic benefits in pre-clinical studies, they have not had widespread use because of conflicting results in clinical trials.

Radiosensitizers

In the 1970s, nitroimidazole derivatives, such as misonidazole, were found to mimic the effect of oxygen in the radiochemical process and examined to see whether enhance the cytotoxic effect of ionizing radiation under hypoxic conditions.[87,88] The theoretically expected oxygen enhancement ratio could be confirmed to be about 1.5–2.0 at a clinically acceptable dose in pre-clinical *in vitro* as well as *in vivo* studies. However, clinical trials with the nitroimidazole derivatives demonstrated limited therapeutic benefit and remain inconclusive. The Radiation Therapy Oncology Group (RTOG) misonidazole trials showed no benefit.[89,90] The misonidazole trial in Denmark also showed no overall benefit. Meanwhile, positive results were also observed in a subgroup of 304 pharyngeal cancer patients, in ENT tumors treated with brachytherapy, and in large ENT tumors treated with hypofractionated external radiation therapy.[91] Moreover, the Danish trial with another radiosensitizer, nimorazole, demonstrated an overall benefit in survival as well as local control.[92] Such an inconclusive outcome of misonidazole was in part due to its dose-limiting toxicities. Effective doses of the drug were found to cause peripheral neuropathy, which is the main reason why it has been kept from routine clinical use. Such a side effect is attributed to the low solubility of misonidazole; therefore, other nitroimidazole derivatives with high solubility, such as etanidazole and

Table 1. Strategies to overcome radioresistance of hypoxic tumor cells.

Strategies	Mechanisms of Action	References
Hyperbaric Oxygenation	direct delivery of molecular oxygen to hypoxic regions	10
Red Blood Cell Transfusion	direct delivery of molecular oxygen to hypoxic regions	84
Erythropoietin Injection	direct delivery of molecular oxygen to hypoxic regions	85
ARCON	direct delivery of molecular oxygen to hypoxic regions and reoxygenation of acute hypoxia	86
nitroimidazole derivatives	radiosensitization by mimicking the effect of molecular oxygen	87–93
Hypoxic Cytotoxins	direct killing of hypoxic tumor cells	3, 94–99
HIF-1 Inhibitors	suppression of radioresistant phenotype of hypoxic tumor cells	7, 18, 100–105
Gene Therapy Strategies	direct killing of hypoxic tumor cells	106–112
Fractionated Radiotherapy	radiation-induced reoxygenation of hypoxic tumor cells	21, 60, 61
IMRT	delivering booster dose of radiation to hypoxic tumor cells	118

doranidazole, have been developed.[93] Etanidazole hardly penetrates into the nervous system tissues and does not cross the blood-brain-barrier, and therefore, shows less neurotoxicity compared with misonidazole. Thus, increased dose of etanidazole can be applicable by a factor of about 3 times than that of misonidazole. Then, positive therapeutic effects were observed in a subset of patients with early nodal disease.

Hypoxic cytotoxins

Although tumor hypoxia is one of the major obstacles in radiation therapy, we can take advantage of it as a tumor-specific therapeutic target and as an alternative to oxygenation of tumor hypoxia.[3] Tirapazamine is a representative hypoxia-activated prodrug which was first described over 20 years ago.[3,94] The hypoxic selectivity of tirapazamine is attributed to its intracellular one-electron reduction to a radical anion which can be reversibly oxidized to the parent nontoxic compound in the presence of molecular oxygen.[95] On the other hand, in the absence of oxygen, the radical anion can be further converted to a toxic hydroxyl radical or to an oxidizing radical through the elimination of water.[96] Both of the resultant radicals damage intracellular macromolecules, causing DNA double-strand breaks (DSBs), single-strand breaks, and base damage, resulting in cytotoxicity. Moreover, Tirapazamine exerts its cytotoxic effect at least in part through poisoning topoisomerase II.[97] Clinical trials with Tirapazamine in combination with radiation have demonstrated benefits in patients with lung cancer or head and neck cancer.[98,99]

HIF-1 inhibitor

Because of its critical function in the radioresistance of hypoxic tumor cells, HIF-1 has been recognized as an excellent molecular target for sensitization of the therapeutic effect of radiation.[3,6,26] YC-1, which was at first synthesized with the aim of activating soluble guanylate cyclase and inhibiting platelet aggregation, was reported to decrease HIF-1α accumulation and HIF-1 target gene expression under hypoxic conditions, thereby inhibiting the growth and spread of tumors.[100,101] More recently, it was reported that YC-1 treatment with optimal timing for the inhibition of radiation-induced activation of HIF-1 significantly delays tumor growth compared to radiation therapy alone.[7,18]

Inhibiting the dimerization of HIF-1α with HIF-1β can be also expected to have radiosensitizing effect because it is required for HIF-1 DNA binding and transcriptional activity. Lee *et al*. identified acriflavine as a drug that binds directly to HIF-1α and inhibits HIF-1 dimerization and transcriptional activity.[102] They reported that acriflavine treatment inhibited intratumoral expression of angiogenic cytokines, mobilization of angiogenic cells into peripheral blood, and tumor vascularization, resulting in the prevention and arrest of tumor growth.[102] Although whether this drug has radiosensitizing effect has not been examined yet, we can expect positive results.

Another approach to HIF-1 inhibition is to negatively regulate the function of key factors which up-regulate the expression or activity of HIF-1. First is the regulation of the PI3K-Akt-mTOR and Ras signaling pathways.[43,47] Mutations in these pathways are commonly seen in human cancers and both of them are known to up-regulate the expression of HIF-1α protein. An mTOR inhibitor, RAD-001, actually decreased the level of HIF-1α protein and its downstream gene products in a mouse model of prostate cancer with high oncogenic Akt activity.[103] Such suppressing effect, at least in part, contributes to the radiosensitizing effect of PI3K-Akt-mTOR signaling pathway inhibitors, RAD-001, LY294002, rapamycin, and wortmannin. Second is the regulation of HSP90 activity. As mentioned above, HSP90 binds to HIF-1α in competition with RACK1 and inhibits the oxygen-independent degradation of HIF-1α. Inhibition of Hsp90 function by 17-allylamino-17-demethoxygeldanamycin or deguelin, a novel natural inhibitor of Hsp90, suppressed an increase in the interaction of HIF-1α with Hsp90 in cancer cells.[104,105] Furthermore, combined treatment with radiation and deguelin significantly decreased the survival and angiogenic potential of radioresistant lung cancer cells *in vitro*.[104]

Gene therapy strategies

Because current gene therapy strategies have poor target specificity, there has been no other way but to directly administer therapeutic genes into tumors. To establish a gene therapy strategy targeting hypoxic/HIF-1-active cancer cells, Harris and his colleagues came up with the idea that HIF-1/HRE-mediated transcriptional initiation is useful to specifically induce therapeutic gene expression in hypoxic regions of solid tumors.[106] Then, extensive efforts have been devoted to the development of artificial HIF-1-dependent promoters, and finally the most effective one, so called 5HRE promoter (5HREp), in which five copies of the HRE enhance transcription from a cytomegalovirus (CMV) minimal promoter, was produced.[64,107] The 5HRE promoter was inserted into upstream of a coding sequence of a suicide gene, which converts a non-toxic prodrug to a toxic drug, with the expectation that the suicide gene expression can be induced in HIF-1-active cancer cells only.[108–112] Combinations of suicide gene/prodrug used in these kinds of studies were herpes simplex virus thymidine kinase (HSV-TK)/ganciclovir (GCV), bacterial cytosine deaminase (BCD)/5-fluorocytosine (5-FC), and bacterial nitroreductase (NTR)/CB1954. The radio-enhancing property as well as tumor growth delay of these strategies was confirmed in an experimental tumor model using a stable transfectant of the 5HREp-suicide gene.[110–112] Moreover, suicide gene therapy using the combination of adenoviral vectors encoding 5HREp-BCD and 5-FC enhanced the effect of fractionated

radiation therapy, opening the door to the clinical application of the strategy.[109)]

Fractionated radiation therapy

Fractionated radiation therapy for cancer has advantages over a single administration of radiation because it can increase therapeutic effect and decrease severe side-effects in normal tissues.[13,80)] In general, these advantages are basically attributed to the sum of positive and negative influences by four parameters; recovery/repair of radiation-induced damage to the cells, redistribution of cell cycle status, reoxygenation of hypoxic cells, and repopulation of surviving cells.[13,80)] These parameters are known as fundamental principles in radiation biology and called "four Rs (4Rs)". Among these four, reoxygenation should be focused on in order to overcome hypoxic tumor cells. It has been reported that the distribution of oxygen from tumor blood vessels to hypoxic tumor cells is dramatically improved after radiotherapy as a result of the death of well oxygenated tumor cells and a subsequent decrease in oxygen consumption there.[60,61)]

In regimens of the most conventional fractionated radiation therapy, 1.8–2.0 Gy of radiation is given per day, about 9.0–10 Gy per week, and up to about 60 Gy for 6 weeks in total. However, such a protocol cannot sufficiently control locally advanced cancers; and therefore, novel schedules have been proposed. Hyperfractionation, in which a smaller dose of radiation such as 1.1–1.2 Gy is delivered twice per day with an interval of about 6 hours, allows the total dose of radiation to be increased and has more therapeutic benefit and fewer side-effects. Accelerated fractionation, in which a relatively high dose of radiation such as 1.5–1.6 Gy is applied twice per day but the total dose is set at the same level as the conventional dose, has an advantage in cases where tumor growth is very rapid because total duration of this treatment is shorter than the other two. Other than these three, various kinds of fractionations were examined in clinical trials and resulted in a significant gain in local control rates.[113,114)] However, from the point of view of reoxygenation, it is critical to understand when and how the reoxygenation occurs, because this information should help us to further optimize the regimens of fractionated radiation therapy.

IMRT

Intensity-modulated radiation therapy (IMRT) is an innovative therapeutic strategy for cancer patients, which enables radiation oncologists to precisely control the distribution of radiation doses specifically to the overall shape of tumors.[115)] The tumor-specificity is accomplished by the development of two key technologies. First is radiological image obtained with computed tomography (CT) and magnetic resonance imaging (MRI), by both of which we can get anatomical information in detail and identify the position of malignant tumors relative to normal structures. Second is the development of multileaf collimators each of which move independently under the control of a computer during radiation therapy so as to allow modulation of the intensity of the photon beam.[116)] Moreover, a combination of multiple external beams realizes a complex three-dimensional planning of dose distribution that conforms to the anatomy of the patients. Thus, IMRT allows the delivery of much higher doses to tumors without increasing the adverse effects on adjacent normal tissue. Such a technological improvement has increased the local control rates of many advanced tumors.[117)] The advent of IMRT makes it possible to give a non-homogenous dose of radiation within a tumor. Namely, attempts have been made to exploit this useful technology to overcome tumor hypoxia, the Hypo-IGRT,; delivering a booster dose of radiation to hypoxic fractions in a malignant tumor.[118)] For that purpose, imaging probes for tumor hypoxia has been extensively developed: *e.g*. CuATSM, ^{18}F-FRP170, and ^{18}F-MISO.

CONCLUSION AND PERSPECTIVE

Based on the accumulated evidence in radiation biology and oncology, there is little doubt that hypoxic tumor cells and HIF-1 active cells are excellent targets to decrease the incidence of both local tumor recurrence and distant tumor metastasis and ultimately patients' mortality. Extensive efforts have been devoted to develop hypoxia-selective cytotoxins/drugs and HIF-1 inhibitors *etc*. Also, development hypoxia image-guided radiation therapy (Hypo-IGRT) aims to deliver a booster dose of radiation to radioresistant fractions. In order to fully gain an effect in combination with radiation therapy, it is critical to monitor the changes in the localization and volume of the radioresistant regions. Because some growth factors and ROS are known to induce HIF-1 activity even under normoxic conditions, imaging strategies for hypoxia (low oxygen conditions) fail to detect cells which are in normoxia but HIF-1-active. Therefore, it is important to develop imaging strategies for both hypoxia and HIF-1 activity. In addition, populations detected by using both strategies should be targeted during radiation therapy.

Optical imaging experiments and immunohistochemical analyses using tumor xenografts revealed that localization and volume of both hypoxic areas and HIF-1-active cells changed during tumor growth and after radiation therapy much more dramatically than we assumed. One important question to be answered is whether the timescale of the dynamics observed in tumor xenografts is the same as that in real human tumors. For this reason, it is necessary to analyze the dynamics of hypoxia and HIF-1-active cells in human cancers. Then, we can optimize the timing and frequency of hypoxia/HIF-1 imaging for the planning of both Hypo-IGRT and chemoradiotherapy with hypoxia/HIF-1-targeted drugs.

ACKNOWLEDGEMENTS

This study was supported by the "Funding Program for Next Generation World-Leading Researchers (NEXT Program)" from the Japan Society for the Promotion of Science (JSPS), by the "Program for Promotion of Fundamental Studies in Health Science" from the National Institute of Biomedical Innovation (NIBIO), Japan, by Grants-in-aid for Scientific Research for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, and by the Sagawa Foundation for the Promotion of Cancer Research.

REFERENCES

1. Rontgen WC (1896) On a New Kind of Rays. Science **3**: 227–231.
2. Bernier J, Hall EJ and Giaccia A (2004) Radiation oncology: a century of achievements. Nat Rev Cancer **4**: 737–747.
3. Brown JM and Wilson WR (2004) Exploiting tumour hypoxia in cancer treatment. Nat Rev Cancer **4**: 437–447.
4. Thomlinson RH and Gray LH (1955) The histological structure of some human lung cancers and the possible implications for radiotherapy. Br J Cancer **9**: 539–549.
5. Bindra RS, Crosby ME and Glazer PM (2007) Regulation of DNA repair in hypoxic cancer cells. Cancer Metastasis Rev **26**: 249–260.
6. Harada H and Hiraoka M (2010) Hypoxia-inducible factor 1 in tumor radioresistance. Current Signal Transduction Therapy **5**: 188–196.
7. Moeller BJ, *et al* (2004) Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: role of reoxygenation, free radicals, and stress granules. Cancer Cell **5**: 429–441.
8. Moeller BJ and Dewhirst MW (2006) HIF-1 and tumour radiosensitivity. Br J Cancer **95**: 1–5.
9. Moeller BJ, *et al* (2005) Pleiotropic effects of HIF-1 blockade on tumor radiosensitivity. Cancer Cell **8**: 99–110.
10. Hirst DG (1986) Oxygen delivery to tumors. Int J Radiat Oncol Biol Phys **12**: 1271–1277.
11. Elkind MM, *et al* (1965) Radiation Response of Mammalian Cells Grown in Culture. V. Temperature Dependence of the Repair of X-Ray Damage in Surviving Cells (Aerobic and Hypoxic). Radiat Res **25**: 359–376.
12. Suit HD (1968) Application of radiobiologic principles to radiation therapy. Cancer **22**: 809–815.
13. Trott KR (1982) Experimental results and clinical implications of the four R's in fractionated radiotherapy. Radiat Environ Biophys **20**: 159–170.
14. Mohan R, *et al* (2000) Radiobiological considerations in the design of fractionation strategies for intensity-modulated radiation therapy of head and neck cancers. Int J Radiat Oncol Biol Phys **46**: 619–630.
15. Harada H, Kizaka-Kondoh S and Hiraoka M (2005) Optical imaging of tumor hypoxia and evaluation of efficacy of a hypoxia-targeting drug in living animals. Mol Imaging **4**: 182–193.
16. Harada H, *et al* (2008) Diameter of tumor blood vessels is a good parameter to estimate HIF-1-active regions in solid tumors. Biochem Biophys Res Commun **373**: 533–538.
17. Harada H, *et al* (2009) The Akt/mTOR pathway assures the synthesis of HIF-1alpha protein in a glucose- and reoxygenation-dependent manner in irradiated tumors. J Biol Chem **284**: 5332–5342.
18. Harada H, *et al* (2009) Treatment regimen determines whether an HIF-1 inhibitor enhances or inhibits the effect of radiation therapy. Br J Cancer **100**: 747–757.
19. Hanahan D and Weinberg RA (2000) The hallmarks of cancer. Cell **100**: 57–70.
20. Vaupel P, Kallinowski F and Okunieff P (1989) Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. Cancer Res **49**: 6449–6465.
21. Hall EJ (1994) Radiobiology for the Radiologists. Fourth Edition, J. B. Lippincott Company, Philadelphia.
22. Brown JM (1979) Evidence for acutely hypoxic cells in mouse tumours, and a possible mechanism of reoxygenation. Br J Radiol **52**: 650–656.
23. Dewhirst MW (2009) Relationships between cycling hypoxia, HIF-1, angiogenesis and oxidative stress. Radiat Res **172**: 653–665.
24. Dewhirst MW, Cao Y and Moeller B (2008) Cycling hypoxia and free radicals regulate angiogenesis and radiotherapy response. Nat Rev Cancer **8**: 425–437.
25. Gray LH, *et al* (1953) The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. Br J Radiol **26**: 638–648.
26. Kizaka-Kondoh S, *et al* (2003) Tumor hypoxia: a target for selective cancer therapy. Cancer Sci **94**: 1021–1028.
27. Aebersold DM, *et al* (2001) Expression of hypoxia-inducible factor-1alpha: a novel predictive and prognostic parameter in the radiotherapy of oropharyngeal cancer. Cancer Res **61**: 2911–2916.
28. Irie N, Matsuo T and Nagata I (2004) Protocol of radiotherapy for glioblastoma according to the expression of HIF-1. Brain Tumor Pathol **21**: 1–6.
29. Ishikawa H, *et al* (2004) Expression of hypoxic-inducible factor 1alpha predicts metastasis-free survival after radiation therapy alone in stage IIIB cervical squamous cell carcinoma. Int J Radiat Oncol Biol Phys **60**: 513–521.
30. Zhang X, *et al* (2004) Enhancement of hypoxia-induced tumor cell death in vitro and radiation therapy in vivo by use of small interfering RNA targeted to hypoxia-inducible factor-1alpha. Cancer Res **64**: 8139–8142.
31. Wang GL, *et al* (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci USA **92**: 5510–5514.
32. Hirota K and Semenza GL (2005) Regulation of hypoxia-inducible factor 1 by prolyl and asparaginyl hydroxylases. Biochem Biophys Res Commun **338**: 610–616.
33. Semenza GL (2001) HIF-1, O(2), and the 3 PHDs: how animal cells signal hypoxia to the nucleus. Cell **107**: 1–3.
34. Jaakkola P, *et al* (2001) Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science **292**: 468–472.

35. Maxwell PH, *et al* (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature **399**: 271–275.
36. Chan DA and Giaccia AJ (2007) Hypoxia, gene expression, and metastasis. Cancer Metastasis Rev **26**: 333–339.
37. Erler JT, *et al* (2006) Lysyl oxidase is essential for hypoxia-induced metastasis. Nature **440**: 1222–1226.
38. Forsythe JA, *et al* (1996) Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol **16**: 4604–4613.
39. Kim JW, Gao P and Dang CV (2007) Effects of hypoxia on tumor metabolism. Cancer Metastasis Rev **26**: 291–298.
40. Rofstad EK (2000) Microenvironment-induced cancer metastasis. Int J Radiat Biol **76**: 589–605.
41. Liu YV, *et al* (2007) RACK1 competes with HSP90 for binding to HIF-1alpha and is required for O(2)-independent and HSP90 inhibitor-induced degradation of HIF-1alpha. Mol Cell **25**: 207–217.
42. Laughner E, *et al* (2001) HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol Cell Biol **21**: 3995–4004.
43. Zundel W, *et al* (2000) Loss of PTEN facilitates HIF-1-mediated gene expression. Genes Dev **14**: 391–396.
44. Feldser D, *et al* (1999) Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2. Cancer Res **59**: 3915–3918.
45. Hellwig-Burgel T, *et al* (1999) Interleukin-1beta and tumor necrosis factor-alpha stimulate DNA binding of hypoxia-inducible factor-1. Blood **94**: 1561–1567.
46. Zelzer E, *et al* (1998) Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1alpha/ARNT. EMBO J **17**: 5085–5094.
47. Zhong H, *et al* (2000) Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. Cancer Res **60**: 1541–1545.
48. Mahon PC, Hirota K and Semenza GL (2001) FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev **15**: 2675–2686.
49. Richard DE, *et al* (1999) p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1alpha (HIF-1alpha) and enhance the transcriptional activity of HIF-1. J Biol Chem **274**: 32631–32637.
50. Sodhi A, *et al* (2000) The Kaposi's sarcoma-associated herpes virus G protein-coupled receptor up-regulates vascular endothelial growth factor expression and secretion through mitogen-activated protein kinase and p38 pathways acting on hypoxia-inducible factor 1alpha. Cancer Res **60**: 4873–4880.
51. Gorski DH, *et al* (1999) Blockage of the vascular endothelial growth factor stress response increases the antitumor effects of ionizing radiation. Cancer Res **59**: 3374–3378.
52. Zeng L, *et al* (2008) TS-1 enhances the effect of radiotherapy by suppressing radiation-induced hypoxia-inducible factor-1 activation and inducing endothelial cell apoptosis. Cancer Sci **99**: 2327–2335.
53. Li F, *et al* (2007) Regulation of HIF-1alpha stability through S-nitrosylation. Mol Cell **26**: 63–74.
54. Ou G, *et al* (2009) Usefulness of HIF-1 imaging for determining optimal timing of combining bevacizumab and radiotherapy. Int J Radiat Oncol Biol Phys **75**: 463–467.
55. Ljungkvist AS, *et al* (2000) Changes in tumor hypoxia measured with a double hypoxic marker technique. Int J Radiat Oncol Biol Phys **48**: 1529–1538.
56. Bennewith KL and Durand RE (2004) Quantifying transient hypoxia in human tumor xenografts by flow cytometry. Cancer Res **64**: 6183–6189.
57. Chaplin DJ, Olive PL and Durand RE (1987) Intermittent blood flow in a murine tumor: radiobiological effects. Cancer Res **47**: 597–601.
58. Ljungkvist AS, *et al* (2007) Dynamics of tumor hypoxia measured with bioreductive hypoxic cell markers. Radiat Res **167**: 127–145.
59. Nehmeh SA, *et al* (2008) Reproducibility of intratumor distribution of (18)F-fluoromisonidazole in head and neck cancer. Int J Radiat Oncol Biol Phys **70**: 235–242.
60. Dorie MJ and Kallman RF (1984) Reoxygenation in the RIF-1 tumor. Int J Radiat Oncol Biol Phys **10**: 687–693.
61. Murata R, *et al* (1996) Reoxygenation after single irradiation in rodent tumors of different types and sizes. Int J Radiat Oncol Biol Phys **34**: 859–865.
62. Harada H, *et al* (2007) The combination of hypoxia-response enhancers and an oxygen-dependent proteolytic motif enables real-time imaging of absolute HIF-1 activity in tumor xenografts. Biochem Biophys Res Commun **360**: 791–796.
63. Liu J, *et al* (2005) Real-time imaging of hypoxia-inducible factor-1 activity in tumor xenografts. J Radiat Res (Tokyo) **46**: 93–102.
64. Shibata T, *et al* (1998) Enhancement of gene expression under hypoxic conditions using fragments of the human vascular endothelial growth factor and the erythropoietin genes. Int J Radiat Oncol Biol Phys **42**: 913–916.
65. Mueller-Klieser W, *et al* (1991) Pathophysiological approaches to identifying tumor hypoxia in patients. Radiother Oncol **20 Suppl 1**: 21–28.
66. Vaupel P, *et al* (1991) Oxygenation of human tumors: evaluation of tissue oxygen distribution in breast cancers by computerized O2 tension measurements. Cancer Res **51**: 3316–3322.
67. Raleigh JA, *et al* (1998) Hypoxia and vascular endothelial growth factor expression in human squamous cell carcinomas using pimonidazole as a hypoxia marker. Cancer Res **58**: 3765–3768.
68. Lord EM, Harwell L and Koch CJ (1993) Detection of hypoxic cells by monoclonal antibody recognizing 2-nitroimidazole adducts. Cancer Res **53**: 5721–5726.
69. Vordermark D and Brown JM (2003) Evaluation of hypoxia-inducible factor-1alpha (HIF-1alpha) as an intrinsic marker of tumor hypoxia in U87 MG human glioblastoma: in vitro and xenograft studies. Int J Radiat Oncol Biol Phys **56**: 1184–1193.
70. Loncaster JA, *et al* (2001) Carbonic anhydrase (CA IX)

expression, a potential new intrinsic marker of hypoxia: correlations with tumor oxygen measurements and prognosis in locally advanced carcinoma of the cervix. Cancer Res **61**: 6394–6399.
71. Cairns RA, Harris IS and Mak TW (2011) Regulation of cancer cell metabolism. Nat Rev Cancer **11**: 85–95.
72. Mankoff DA, *et al* (2007) Tumor-specific positron emission tomography imaging in patients: [18F] fluorodeoxyglucose and beyond. Clin Cancer Res **13**: 3460–3469.
73. Semenza GL (2009) Regulation of cancer cell metabolism by hypoxia-inducible factor 1. Semin Cancer Biol **19**: 12–16.
74. Chen C, *et al* (2001) Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. J Biol Chem **276**: 9519–9525.
75. Lopez-Lazaro M (2008) The warburg effect: why and how do cancer cells activate glycolysis in the presence of oxygen? Anticancer Agents Med Chem **8**: 305–312.
76. Evans SM, *et al* (2000) Noninvasive detection of tumor hypoxia using the 2-nitroimidazole [18F]EF1. J Nucl Med **41**: 327–336.
77. Fujibayashi Y, *et al* (1997) Copper-62-ATSM: a new hypoxia imaging agent with high membrane permeability and low redox potential. J Nucl Med **38**: 1155–1160.
78. Rasey JS, *et al* (1987) Characterization of radiolabeled fluoromisonidazole as a probe for hypoxic cells. Radiat Res **111**: 292–304.
79. Wang K, *et al* (2009) Modeling acute and chronic hypoxia using serial images of 18F-FMISO PET. Med Phys **36**: 4400–4408.
80. Egeland TA, *et al* (2006) Assessment of fraction of radiobiologically hypoxic cells in human melanoma xenografts by dynamic contrast-enhanced MRI. Magn Reson Med **55**: 874–882.
81. Magat J, *et al* (2010) Noninvasive mapping of spontaneous fluctuations in tumor oxygenation using 19F MRI. Med Phys **37**: 5434–5441.
82. Matsumoto S, *et al* (2010) Imaging cycling tumor hypoxia. Cancer Res **70**: 10019–10023.
83. Yasui H, *et al* (2010) Low-field magnetic resonance imaging to visualize chronic and cycling hypoxia in tumor-bearing mice. Cancer Res **70**: 6427–6436.
84. Poskitt TR (1987) Radiation therapy and the role of red blood cell transfusion. Cancer Invest **5**: 231–236.
85. Pinel S, *et al* (2004) Erythropoietin-induced reduction of hypoxia before and during fractionated irradiation contributes to improvement of radioresponse in human glioma xenografts. Int J Radiat Oncol Biol Phys **59**: 250–259.
86. Kaanders JH, Bussink J and van der Kogel AJ (2002) ARCON: a novel biology-based approach in radiotherapy. Lancet Oncol **3**: 728–737.
87. Coleman CN (1985) Hypoxic cell radiosensitizers: expectations and progress in drug development. Int J Radiat Oncol Biol Phys **11**: 323–329.
88. Stratford IJ (1982) Mechanisms of hypoxic cell radiosensitization and the development of new sensitizers. Int J Radiat Oncol Biol Phys **8**: 391–398.
89. Minsky BD and Leibel SA (1989) The treatment of hepatic metastases from colorectal cancer with radiation therapy alone or combined with chemotherapy or misonidazole. Cancer Treat Rev **16**: 213–219.
90. Simpson JR, *et al* (1989) Radiation therapy alone or combined with misonidazole in the treatment of locally advanced non-oat cell lung cancer: report of an RTOG prospective randomized trial. Int J Radiat Oncol Biol Phys **16**: 1483–1491.
91. Baillet F, *et al* (1989) Positive clinical experience with misonidazole in brachytherapy and external radiotherapy. Int J Radiat Oncol Biol Phys **16**: 1073–1075.
92. Overgaard J, *et al* (1998) A randomized double-blind phase III study of nimorazole as a hypoxic radiosensitizer of primary radiotherapy in supraglottic larynx and pharynx carcinoma. Results of the Danish Head and Neck Cancer Study (DAHANCA) Protocol 5-85. Radiother Oncol **46**: 135–146.
93. Yahiro T, *et al* (2005) Effects of hypoxic cell radiosensitizer doranidazole (PR-350) on the radioresponse of murine and human tumor cells in vitro and in vivo. J Radiat Res (Tokyo) **46**: 363–372.
94. Zeman EM, *et al* (1986) SR-4233: a new bioreductive agent with high selective toxicity for hypoxic mammalian cells. Int J Radiat Oncol Biol Phys **12**: 1239–1242.
95. Baker MA, *et al* (1988) Metabolism of SR 4233 by Chinese hamster ovary cells: basis of selective hypoxic cytotoxicity. Cancer Res **48**: 5947–5952.
96. Zagorevskii D, *et al* (2003) A mass spectrometry study of tirapazamine and its metabolites. insights into the mechanism of metabolic transformations and the characterization of reaction intermediates. J Am Soc Mass Spectrom **14**: 881–892.
97. Peters KB and Brown JM (2002) Tirapazamine: a hypoxia-activated topoisomerase II poison. Cancer Res **62**: 5248–5253.
98. Rischin D, *et al* (2005) Tirapazamine, Cisplatin, and Radiation versus Fluorouracil, Cisplatin, and Radiation in patients with locally advanced head and neck cancer: a randomized phase II trial of the Trans-Tasman Radiation Oncology Group (TROG 98.02). J Clin Oncol **23**: 79–87.
99. von Pawel J, *et al* (2000) Tirapazamine plus cisplatin versus cisplatin in advanced non-small-cell lung cancer: A report of the international CATAPULT I study group. Cisplatin and Tirapazamine in Subjects with Advanced Previously Untreated Non-Small-Cell Lung Tumors. J Clin Oncol **18**: 1351–1359.
100. Shin DH, *et al* (2007) Preclinical evaluation of YC-1, a HIF inhibitor, for the prevention of tumor spreading. Cancer Lett **255**: 107–116.
101. Yeo EJ, *et al* (2003) YC-1: a potential anticancer drug targeting hypoxia-inducible factor 1. J Natl Cancer Inst **95**: 516–525.
102. Lee K, *et al* (2009) Acriflavine inhibits HIF-1 dimerization, tumor growth, and vascularization. Proc Natl Acad Sci USA **106**: 17910–17915.
103. Majumder PK, *et al* (2004) mTOR inhibition reverses Akt-dependent prostate intraepithelial neoplasia through regulation of apoptotic and HIF-1-dependent pathways. Nat Med **10**: 594–601.
104. Kim WY, *et al* (2009) Targeting heat shock protein 90 overrides the resistance of lung cancer cells by blocking radi-

ation-induced stabilization of hypoxia-inducible factor-1alpha. Cancer Res **69**: 1624–1632.
105. Oh SH, *et al* (2007) Structural basis for depletion of heat shock protein 90 client proteins by deguelin. J Natl Cancer Inst **99**: 949–961.
106. Dachs GU, *et al* (1997) Targeting gene expression to hypoxic tumor cells. Nat Med **3**: 515–520.
107. Shibata T, Giaccia AJ and Brown JM (2000) Development of a hypoxia-responsive vector for tumor-specific gene therapy. Gene Ther **7**: 493–498.
108. Greco O, *et al* (2003) How to overcome (and exploit) tumor hypoxia for targeted gene therapy. J Cell Physiol **197**: 312–325.
109. Liu J, *et al* (2007) Adenovirus-mediated hypoxia-targeting cytosine deaminase gene therapy enhances radiotherapy in tumour xenografts. Br J Cancer **96**: 1871–1878.
110. Ogura M, *et al* (2005) A tumor-specific gene therapy strategy targeting dysregulation of the VHL/HIF pathway in renal cell carcinomas. Cancer Sci **96**: 288–294.
111. Patterson AV, *et al* (2002) Oxygen-sensitive enzyme-prodrug gene therapy for the eradication of radiation-resistant solid tumours. Gene Ther **9**: 946–954.
112. Shibata T, Giaccia AJ and Brown JM (2002) Hypoxia-inducible regulation of a prodrug-activating enzyme for tumor-specific gene therapy. Neoplasia **4**: 40–48.
113. Horiot JC, *et al* (1992) Hyperfractionation versus conventional fractionation in oropharyngeal carcinoma: final analysis of a randomized trial of the EORTC cooperative group of radiotherapy. Radiother Oncol **25**: 231–241.
114. Thames HD Jr, *et al* (1982) Changes in early and late radiation responses with altered dose fractionation: implications for dose-survival relationships. Int J Radiat Oncol Biol Phys **8**: 219–226.
115. Yu CX (1995) Intensity-modulated arc therapy with dynamic multileaf collimation: an alternative to tomotherapy. Phys Med Biol **40**: 1435–1449.
116. Aoki Y, *et al* (1987) An integrated radiotherapy treatment system and its clinical application. Radiat Med **5**: 131–141.
117. Staffurth J (2010) A review of the clinical evidence for intensity-modulated radiotherapy. Clin Oncol (R Coll Radiol) **22**: 643–657.
118. Lecchi M, *et al* (2008) Current concepts on imaging in radiotherapy. Eur J Nucl Med Mol Imaging **35**: 821–837.

Received on April 11, 2011
Revision received on June 6, 2011
Accepted on July 5, 2011