difficult, and automated solid-phase synthesis is possible. Moreover, they have flexible sites for covalent attachment to other molecules. Hairpin polyamides linked with an alkylating agent may broaden the targets of transcriptional regulation of coding sequences [17]. In fact, we have successfully inhibited gene transcription of the green fluorescent protein and luciferase by alkylating hairpin polyamides [18].

Inhibiting the HIF transcription pathway is a fascinating strategy for cancer control, especially in the case of renal cell carcinomas. HIF controls genes related to the progression of cancers. Genes for platelet-derived growth factor (PDGF), VEGF, epidermal growth factor (EGF), and transforming growth factor-1α (TGF-1α are among those regulated by HIF-1 (a dimer of HIF-1α and ARNT). Therapeutic approaches using each of these molecules are being trialled clinically [19]. Avastin™ (anti-VEGF antibody), SU11248 (a small molecule targeting the tyrosine kinase domain of the VEGF receptor), and Tarceva™ or Iressa™ (molecules targeting the tyrosine kinase domain of the EGF receptor) are currently being investigated as potential agents against advanced renal cell carcinoma. Recently, we have shown that glucocorticoids can down-regulate VEGF in renal cell carcinoma cells [20]. Suppressing the binding of HIF-1 to the HRE may be a useful strategy for inhibition of these molecules. In addition, we and several other groups have demonstrated that HIF-2α, another α subunit of HIF, is more critical than HIF-1α in renal cell cancer [10,21]. As HIF-2 (a dimer of HIF-2α and ARNT) shares an HRE with HIF-1, targeting the HIF-HRE interaction may be more useful in the management of renal cell carcinoma than using the molecular therapeutic drugs described earlier. In the current study, the polyamide mixture successfully suppressed transcription induced not only by HIF-1α but also by HIF-2α. Our findings may facilitate the realization of the "transcription therapy" concept proposed by Dervan [5] for cancer treatment.

Conclusions

VEGF transcription was successfully suppressed by a combination of three Py-Im hairpin polyamides targeting the HRE. The use of Py-Im hairpin polyamides may be a new strategy for the treatment of renal cell carcinoma.

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Review Article

Antitumor Protein Therapy; Application of the Protein Transduction Domain to the Development of a Protein Drug for Cancer Treatment

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The genomic information obtained through the human genome project has been accelerating the analysis of the functions of various disease relevant genes. The high molecular weight biomolecules, including oligonucleotides, antisense nucleotides, small interference RNA and peptides, as well as genes (cDNA) and proteins, are becoming increasingly important for the development of molecular therapies. However, the potential of such information-rich macromolecules for therapeutic use has been limited by the poor permeability across the lipid bilayer of the cellular plasma membrane. Over the past decade, a unique activity of oligopeptides, known as protein transduction domains (PTDs) or cell penetrating peptides (CPPs), has made it possible to transduce biologically active macromolecules into living cells *in vitro* by conjugating a PTD to the desired macromolecule. Furthermore, this activity has also enabled the systemic delivery of bioactive macromolecules to all tissues in living animals. However, we are now confronted with the next difficulty delivering the macromolecules specifically to the therapeutic targets *in vivo*. In this review, we focus on the application of PTD to develop antitumor macromolecules and introduce several representative strategies to discriminate between tumor and normal tissue. In addition, we discuss the unique characteristics of breast cancer, which are expected to facilitate the application of PTD to develop novel protein therapy for breast cancer.

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Key words: Protein therapy, Protein transduction domain (PTD), HIV-1 tat, Tumor hypoxia, Hypoxia-inducible factor-1 (HIF-1)

Efficient internalization of therapeutic agents into target cells is critical to gain the desired therapeutic effect. However, since the plasma membrane of the cell surface forms an effective barrier and limits the internalization of high molecular weight materials into the cells, the application of information-rich macromolecules, such as DNA and proteins, to therapies has been restricted. A variety of methods have been widely proposed to effect their delivery into living cells *in vivo* as well as *in vitro*[1-3]; unfortunately, many of them have shown inefficient delivery. In these strategies, a number of other problems, such as complex manipulation, cellular toxicity and immunogenicity, have been reported and have prevented macromolecules from routine therapeutic use.

In 1988, Green *et al.* and Frankel *et al.* separately reported that the transcriptional activator of transcription (TAT) protein from human immunodeficiency virus-1 (HIV-1) has a unique potential to enter cells in culture when added exogenously[4,5]. The domain responsible for this translocation has been ascribed to the short basic region comprised by residues 47-57 of the TAT protein and is termed the "TAT protein transduction domain (PTD)"[6]. Subsequent studies have demon-

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Abbreviations:
PTD, Protein transduction domain; CPP, Cell penetrating peptide; HIF, Hypoxia-inducible factor; TAT, Transcriptional activator of transcription; HIV-1, Human immunodeficiency virus-1; IGF-I, Insulin-like growth factor-I; IGF-IR, IGF-I receptor; RCC, Renal cell carcinoma; VHL, Von Hippel-Lindau; CTL, Cytotoxic T lymphocyte; DC, Dendritic cell; OVA, Ovalbumin; TRP2, Tyrosinase-related protein 2; IAP, Inhibitors of apoptosis protein; Smac, Second mitochondria-derived activator of caspases; XIAP, X-linked IAP; TRAIL, Tumor necrosis factor-related apoptosis-inducing ligand; ODD, Oxygen-dependent degradation; β-Gal, β-galactosidase; TOP3, TAT-ODD-Procaspase-3; AI, Apoptosis index; pO_2, Oxygen partial pressure; RNAP, RNA polymerase

strated that TAT-PTD facilitates the internalization of conjugated proteins into living cells *in vitro*[6]. Likewise, a number of the other cationic peptides, e.g. the peptides from the third α helix of the antennapedia homeodomain and from the VP22 protein of the herpes simplex virus, have been reported as PTDs showing the same attractive activity as TAT-PTD[7]. The common feature among these peptides is their highly cationic nature, which is due to their high proportion of basic amino acids, such as arginine and lysine residues[8]. Using these PTDs, various kinds of physiologically and therapeutically active macromolecules, such as peptides, proteins[9], oligo DNAs[10], super magnet beads[11], liposomes[1] and λ phages[12] have been successfully transduced into living cells. Intracellular delivery of these macromolecules modulates the functions of various genes related to the cell cycle[13] and apoptosis[14] *in vitro*. Moreover, Schwarze *et al.* demonstrated that intraperitoneal injection of a TAT-PTD-fused 120 kDa β-galactosidase (β-Gal) protein resulted in the delivery of the biologically active fusion protein to all tissues in mice, including the brain[15]. Their results opened a new possibility for the direct delivery of macromolecules into patients as protein therapy. Indeed, several groups, including ours, have applied this strategy to develop novel protein drugs to treat preclinical tumor-bearing animals[16-20].

In this review, we describe the properties and the potential of TAT-PTD as a carrier of information-rich macromolecules, and introduce representative research, in which TAT-PTD-mediated protein therapy showed significant antitumor effects with target-specificity, but without side effects. Furthermore, we discuss the possibilities of such protein therapy for breast cancer treatment.

Mechanism of TAT-PTD-Mediated Protein Transduction into Living Cells

Despite the distinctive potential of the TAT-PTD and the other arginine- and/or lysine-rich peptides as carriers of macromolecules, little is known about the mechanism involved in the cellular uptake of PTD-fused macromolecules as well as the wild type HIV-1 TAT protein.

In the early days, it was reported that no inhibition of internalization was observed at 4 ℃[21], and similar observations were reported for the basic amino acid-rich peptide derived from the antennapedia homeodomain[22]. Therefore, until recently, it was widely assumed that the PTD-mediated internalization of macromolecules occurs in an energy- and receptor-independent manner and is alternatively based on direct transport through the lipid bilayer[21]. However, it has been reported that the energy- and receptor-independence of PTD-mediated internalization are due to experimental artifacts in the process of cell fixation prior to microscopic observation and also due to the inadequate removal of proteins bound to the cell surface[23, 24]. Furthermore, it has been reported that the internalization is almost completely suppressed at 4 ℃ in unfixed conditions[24, 25]. These results, together with the observation that heparan sulfate and the inhibitor of low density lipoprotein receptor-related protein precluded the cellular uptake of PTD-fused macromolecules[25, 26], suggest that the interaction of TAT-PTD with cell surface constituents plays an important role, and is followed by an active endocytic process. Several recent papers support the involvement of an endocytic pathway in the PTD-mediated protein internalization[27, 28].

Since endocytosis is a complex mechanism including several different pathways, the identification of the critical pathway responsible for the internalization has recently been commenced. Using a permeable TAT-Cre recombinase reporter assay on living cells, Wadia *et al.* extensively analyzed the mechanism of cellular uptake of TAT-fusion protein and clearly summarized the details in 2004. After the initial ionic cell-surface interaction, TAT-fusion proteins are rapidly internalized by lipid raft-dependent macropinocytosis, but are independent of interleukin-2 receptor/raft- caveolar- and clathrin-mediated endocytosis and phagocytosis[29]. On the other hand, Richard *et al.* demonstrated in 2005 that a specific inhibitor of clathrin-dependent endocytosis partially inhibits TAT peptide uptake, implicating this pathway in TAT-peptide entry[30]. The molecular basis for the PTD-mediated cellular uptake of macromolecules into living cells still remains controversial, so further study is necessary to fully understand the process.

Development of TAT-PTD-Mediated Antitumor Protein Therapies

Research on protein transduction has dramatically expanded from *in vitro* to *in vivo* in the last decade. The advantage of this application is that we can accomplish rapid and equal distribution of PTD-linked macromolecules to all tissues and

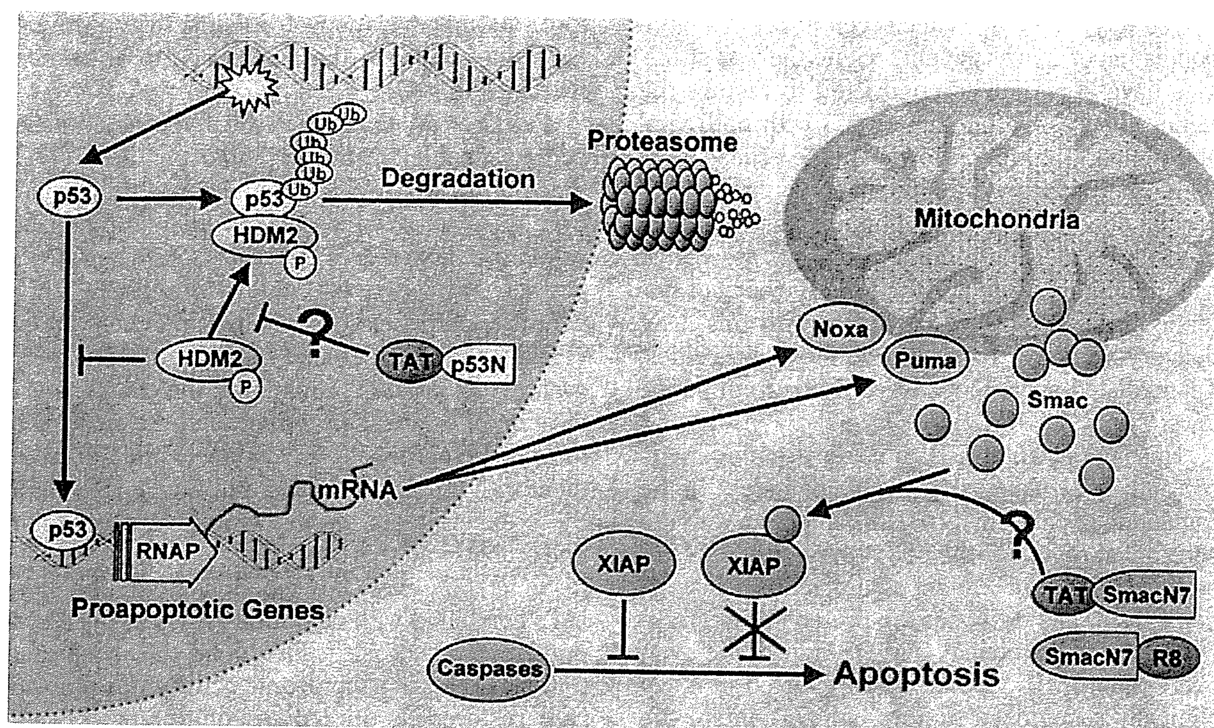

Fig 1. p53-related cellular apoptosis. HDM2 facilitates the proteolytic degradation of p53 protein. However, once damages of genomic DNA become severe, p53 is stabilized and induces various gene expressions such as Noxa and Puma and induce the release of Smac protein from mitochondria. The Smac protein interacts with XIAP and suppresses its activity, resulting in apoptosis. Since overexpression of HDM2 and IAPs inhibits apoptosis in many clinical tumors, the inhibition of their activities by p53N peptide and SmacN7 peptide respectively have been examined as novel antitumor strategies.

cells *in vivo*. However, it conversely leads to disadvantages in others. Especially for the development of PTD-fused anticancer macromolecules, the medications should have target-specificity and act locally, otherwise it may lead to damage of normal tissues and result in side effects. In the following sections, we describe representative applications showing target-specificity as well as the antitumor effect of TAT-mediated protein therapies *in vivo*.

1) Application of a Tumor Suppressor Gene, p53

Accumulated knowledge about signal cascades in cancer cells has revealed that genetic alterations of oncogenes and/or tumor suppressor genes make cells more malignant, resulting in deregulated proliferation and the evasion of apoptosis. In the development of novel cancer therapy, significant efforts at restoring the lesions that prevent the implementation of the apoptotic response have been made in order to specifically cause the death of malignant cells and in order to spare normal cells carrying few such apoptotic burdens. Such a strategy has been expected to show a much lower toxicity in normal tissue, compared with the conventional genotoxic agents that are currently in clinical use.

The gene encoding the tumor suppressor p53 is the most common anti-apoptotic lesion in cancer cells[31] and approximately 50% of human cancers bear p53 gene mutations. In most remaining cases, p53 activity is impaired by alternative molecular mechanisms, such as an elevated level of a p53 inhibitor, Mdm2[32] and the E6 protein of HPV[33], or silencing of a p53 co-activator, ARF[34,35]. One of the most important functions of p53 is "cell cycle arrest", in which p53 disturbs the replication of damaged genomic DNA and the fixation of mutations, allowing for DNA repair. Another important function is the "induction of apoptosis", which occurs in cases in which the damage to the genomic DNA is too severe to be repaired (Fig 1). These abilities of p53 are essential for the proper regulation of cell proliferation in multi-cellular organisms[35]. Loss of these functions frequently leads to cellular neoplastic transformation, and increases the resistance of cancer cells to anti-cancer therapies[36]. Therefore, restoring p53 activity in tumor cells has been expected to be an effec-

tive strategy to induce cancer cell death in a large population of cancer patients. Gene therapy strategies have been indeed conducted to restore the tumor suppressor function of p53 with both viral and non-viral vectors. However, the efficacies of these approaches were difficult to confirm under certain conditions in clinical studies as well as in preclinical studies[37, 38]. Some problems associated with immunogenicity and the low efficiency of systemic distribution were inevitable with this drug delivery system[39]. To overcome these difficulties, Tat-mediated approaches were carried out as follows.

Harbour *et al.* aimed to restore endogenous p53 activity by using a permeable peptide[18]. In the regulation of p53 activity, HDM2 interacts with the N-terminal region of the p53 protein and decreases the ability of p53 to act as a positive transcriptional factor and facilitates the proteolytic degradation of the p53 protein[40] (Fig 1). Indeed, the over-expression of HDM2 has been reported in many clinically recognized tumors, which contain the wild type p53 gene, and is associated with the functional inactivation of the p53 protein[41-43]. Therefore, it is anticipated that the disruption of the inhibitory effect of HDM2 on p53 activity would yield therapeutic benefits in tumor cells that over-express the HDM2 protein. To examine this hypothesis, the N-terminal region of the p53 protein was fused to the TAT-PTD. The resultant TAT-p53N peptide induced the rapid accumulation of p53 and the activation of apoptotic genes, and resulted in the preferential killing of tumor cells and the regression of human retinoblastoma cells in a rabbit eye[18]. Minimal retinal damage was observed after intravitreal injection[18].

2) Application of a Proapoptotic Gene, Smac

A major obstacle in cancer therapy is the resistance of cancer cells to current anticancer treatments, such as chemotherapy and radiotherapy[44]. Defects in apoptotic programs are caused by deregulated expression and function of the components of the apoptotic pathway and contribute to such resistance[45, 46]. Inhibitors of apoptosis proteins (IAPs) are frequently overexpresssed in malignant tumors[47], and they inhibit caspase activity by directly binding to activated caspase-3 and-7[48, 49]. The second mitochondria-derived activator of caspases (Smac) was identified as the protein that is released from the mitochondria to the cytosol in response to apoptotic stimuli and antagonizes IAPs to promote apoptosis[50, 51] (Fig 1). Therefore, the up-regulation of Smac activity in tumor cells may improve the resistance to anticancer therapies (Fig 1).

Fulda *et al.* examined the hypothesis using cell-permeable synthetic Smac peptides (TAT-SmacN7 in this review) containing a polypeptide from the N-terminal of Smac protein for the inactivation of X-linked IAP (XIAP)[52, 53]. As a result, the peptide enhanced the ability of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in an intracranial malignant glioma xenograft model *in vivo*[52]. Moreover, the complete eradication of established tumors and the survival of mice were achieved only upon combined treatment with the Smac peptide and Apo2L/TRAIL. In these experiments, no detectable toxicity to normal brain tissue was observed.

Yang *et al.* examined whether the inhibition of IAPs combined with chemotherapy produced synergistic effects or not[19]. First of all, they confirmed that the defect in apoptosome activity was dramatically restored by the IAP-targeting SmacN7 peptide, which is the seven N-terminal amino residues of mature Smac and has the potential to disrupt XIAP-caspase-9 interaction. On the other hand, SmacN7 peptide did not show any striking effect on the apoptosome activity of normal lung fibroblast cells. They finally demonstrated that newly synthesized SmacN7 peptide fused to the cell membrane permeable polyarginine (SmacN7R8) strongly reversed the apoptosis resistance, and displayed a synergistic effect with chemotherapy *in vivo*.

3) Application of a Tumor Suppressor Gene, VHL

Since it was reported that the growths of a variety of cancer cells are dependent on insulin-like growth factor-I (IGF-I)-mediated signaling, inhibiting the pathway has shown therapeutic effects on a variety of experimental tumor xenografts (Fig 2). For example, a truncated form of the IGF-I receptor (IGF-IR) acts as a dominant negative inhibitor of IGF-IR and abrogates ligand-dependent cellular transformation and tumorigenesis *in vitro* and *in vivo*[54]. Similarly, a specific IGF-IR antibody[55] and a specific IGF-IR antisense oligonucleotide[56] suppress the tumor growth and prolong the survival of tumor-bearing mice, respectively. Renal cell carcinoma (RCC) was reported to be dependent on the IGF-I-mediated signaling pathway for its growth. Previously, Datta *et al.* reported that IGF-I-mediated signaling is inhibited in the

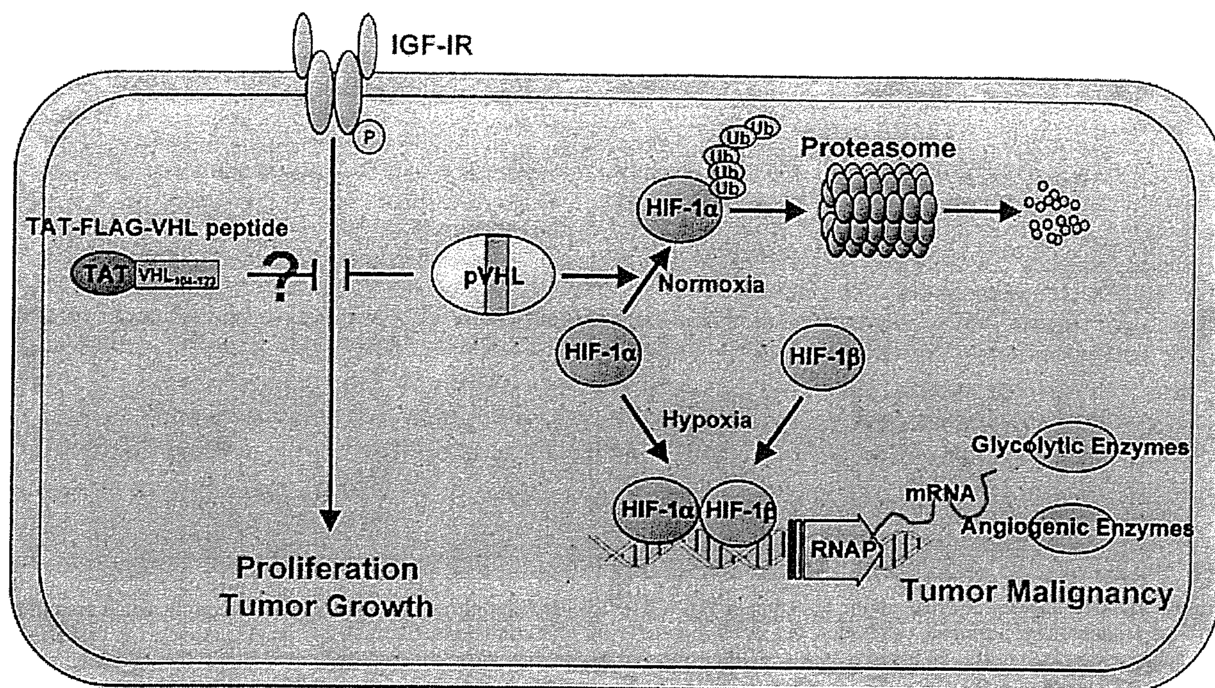

Fig 2. Function of pVHL in IGF-I-mediated signaling and in hypoxia-responsible gene expression. The growth of various cancer cells are dependent on IGF-I signaling. The signal is inhibited in the presence of pVHL in RCC cells, and thus inhibition of this signal pathway has been aimed by $VHL_{104\text{-}123}$ peptide. pVHL also acts on hypoxia-dependent gene expression. In normoxic conditions, hydroxylated HIF-1α protein is recognized by pVHL and ubiquitinated, resulting in the rapid proteolytic degradation. In hypoxic conditions, stabilized HIF-1α interacts with constitutively expressed HIF-1β and induces various gene expressions related to tumor malignancy.

presence of wild type von Hippel-Lindau tumor suppressor gene product (pVHL) in RCC cells and a specific amino acid sequence (104-123) in the β domain of the pVHL ($VHL_{104\text{-}123}$) is responsible for this function[57] (Fig 2). These results indicate that the pVHL function via the 104-123 amino acid region leads to the restricted IGF-IR signaling, resulting in restricted cell proliferation and restricted RCC growth. This is consistent with the reports that the VHL tumor suppressor gene is mutated in the majority of patients with RCCs, as well as in patients with VHL disease[58, 59], and that the mutations are located in one of the hotspots of the VHL gene[60]. In addition to a well-known function of pVHL to regulate the stability of hypoxia-inducible factor-1α (HIF-1α) protein[61], this activity must also play an important role in the tumorigenesis of RCCs.

Based on this preclinical research, Datta *et al.* examined the effects of $VHL_{104\text{-}123}$ on tumor characteristics[20] (Fig 2). $VHL_{104\text{-}123}$ conjugated to the TAT-PTD (TAT-FLAG-VHL peptide) inhibited the thymidine incorporation into RCC cells by nearly 80% compared with a counterpart protein (TAT-FLAG). Furthermore, the TAT-FLAG-VHL peptide inhibited the tyrosine phosphorylation of MAP kinase, an essential downstream molecule that leads to cell proliferation. Thus, these results suggest that TAT-FLAG-VHL peptide blocks the IGF-I-induced RCC proliferation *in vitro*. Furthermore, i.p. injection of TAT-FLAG-VHL peptide retarded the growth of subcutaneous RCC tumors, and in some cases, regressed the tumors volume, and dramatically inhibited the invasiveness deeper into the muscle layer.

4) Application of a HIF-1α ODD Domain; Development of Hypoxia-Targeting Protein Drug

The genetic alterations in tumor cells directly cause the deregulated proliferation and the high metabolic demands of tumor cells, which in turn lead to the development of hypoxia in solid tumors[62, 63]. Tumor hypoxia has been recognized as a tumor specific microenvironment, in other words, healthy adults probably have few such tissues. In such conditions, a transcriptional factor, hypoxia-inducible factor-1 (HIF-1), induces various genes related to angiogenesis[64] and glycolysis[65], and leads to invasive and metastatic properties in

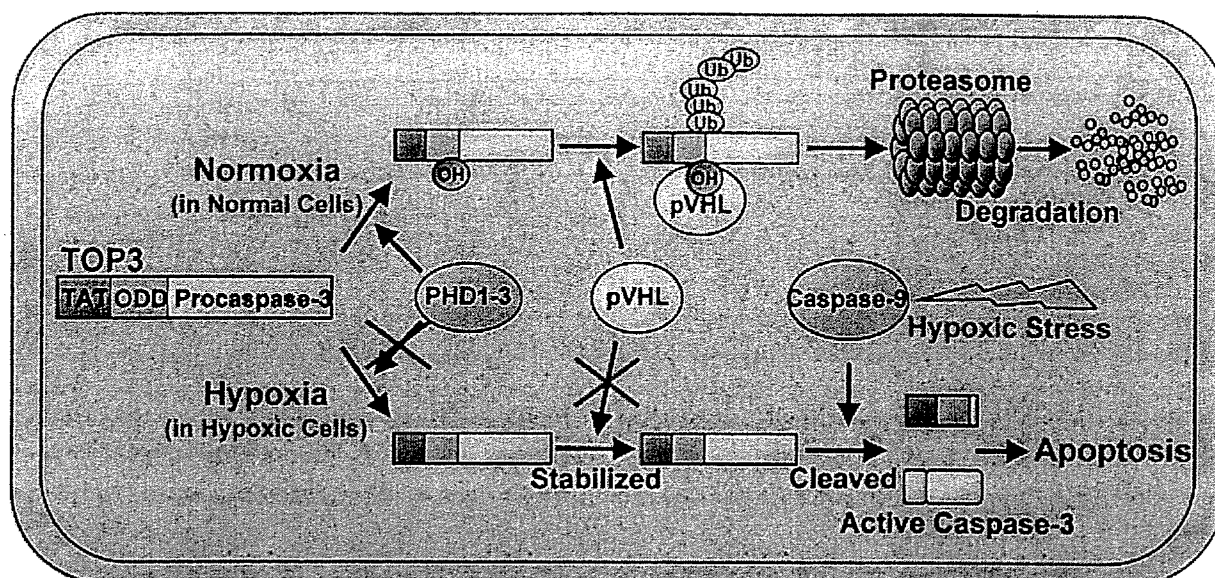

Fig 3. Hypoxia-dependent Proapoptotic Function of TOP3. TOP3 is degraded through the same ubiquitin-proteasome system as HIF-1α protein under normoxic conditions, but stabilized under hypoxic conditions. Because upstream caspases, *e.g.* caspases-9, are activated to some extent by hypoxic stress, TOP3 is cleaved to generate an active caspase-3, resulting in the enhancement of apoptotic cell death.

tumor cells[66]. HIF-1 activity is associated with the resistance of tumor cells to conventional radiotherapy and chemotherapy[67,68] and with the patient mortality in clinical studies[69-71]. Therefore, extensive efforts have been directed toward the development of novel therapies, which specifically damage the hypoxic/HIF-1-activating tumor cells[68,72].

HIF-1 is a heterodimeric transcriptional factor composed of an alpha subunit (HIF-1α) and a constitutively expressed beta subunit (HIF-1β)[73] (Fig 2). HIF-1α expression is tightly regulated at the post-translational level by oxygen-dependent prolyl hydroxylation and subsequent ubiquitination of its oxygen-dependent degradation (ODD) domain within the HIF-1α protein[61]. The pVHL is responsible for the ubiquitination. The stability of the HIF-1α protein is mainly responsible for the regulation of HIF-1 transcriptional activity[73].

We applied this unique property of the ODD domain to develop a novel hypoxia-targeting protein drug[16]. First of all, we identified the minimum region of the ODD domain responsible for the oxygen-dependent degradation of arbitrary proteins fused to it. As a result, we confirmed the hypoxia-dependent β-Gal and luciferase activity of ODD-β-Gal fusion protein[16] and ODD-Luciferase fusion protein (Harada *et al.*, in preparation), respectively, in the culture cell[16]. To apply the ODD-fusion protein to an *in vivo* study, we fused TAT-PTD to the N-terminal of the ODD-β-Gal protein and created a TAT-ODD-β-Gal triple fusion protein. After i.p. injection with the TAT-ODD-β-Gal fusion protein to subcutaneous tumor-bearing mice, the β-Gal activity and the existence of the fusion protein were detected only in the hypoxic regions of the solid tumor. On the other hand, they were not observed in the normal tissue. These results demonstrate that biologically active proteins can be exogenously delivered to hypoxic tumor cells by the TAT-ODD peptide *in vivo*. This was the first example demonstrating the target-specificity of TAT-mediated protein delivery. To examine whether the TAT-ODD fusion protein with cytotoxicity shows antitumor effects or not, the TAT-ODD peptide was further fused to a proapoptotic protein (Fig 3). We intentionally chose a precursor of caspase-3, procaspase-3, because it is activated in response to hypoxic stress, which was thought to reduce the possibility of side effects in the well-oxygenated normal tissues (Fig 3). Systemic administration with the resultant fusion protein, TAT-ODD-Procaspase-3 (TOP3), reduced the tumor mass as well as suppressed the tumor growth without any obvious side effects in tumor-bearing mice. The hypoxia-targeting effect of TOP3 was proven using a rat ascites model, in which intraperitoneal injection with MM1 cells results in highly hypoxic ascetic fluid[17]. Inoue *et*

al. demonstrated that intraperitoneal injection with TOP3 resulted in a significant increase in the lifespan of rats with the malignant ascites, and furthermore, 60% of the treated animals were cured without the recurrence of ascites.

5) Other Possibilities for the Development of TAT-PTD-Mediated Antitumor Protein Therapy

Several *in vitro* studies have reported other possibilities which may enable delivery of the biologically active macromolecule specifically to the desired tumor *in vivo*[74, 75]. First, the fact that PTDs selectively interact with distinct glycosaminoglycan species may allow targeting of selective tissues that differ in their surface-expressed glycosaminoglycan patterns[27]. Second, by inserting a tissue- and organelle-specific cleavage recognition site between PTD and the macromolecule, PTD may be cleaved off, resulting in the accumulation of the PTD-free macromolecules in the desired tissue and organelle, respectively[76]. Third, it is also possible to generate a PTD-linked protein drug that specifically acts in tumor cells while not affecting normal cells, by applying transformed cell-specific protein activity[77]. Finally, by using a peptide that can be recognized by the tumor-specific membrane proteins, it may be possible to design a variety of proteins that specifically internalize into desired tissues.

Application of TAT-Mediated Protein Drugs to Breast Cancer Treatments

Low molecular weight chemical compounds easily pass through the cellular plasma membrane *in vitro*, and furthermore show efficient distribution *in vivo*, and thus they have been focused on for a long time in the development of anticancer drugs. Almost all of the conventional chemotherapeutic agents, however, show low target-specificity and largely affect normal tissues as well as tumors. TAT-PTD has also been reported to transduce various macromolecules to all tissues *in vivo*[15], so this technique may cause side effects, but for additional devices. Over the past decade, several modifications have been examined to achieve a tumor-specific antitumor effect of PTD-mediated strategies, as mentioned above. To apply TAT-mediated protein therapy to breast cancer treatments, it is necessary to understand the common features among breast cancers and utilize them to construct a protein drug targeting breast cancer. In the remainder of the present review, we will discuss the characteristics of breast cancer and the possibilities of PTD-mediated protein therapy targeting then.

Pusztai *et al.* identified a number of novel and routine prognostic markers of breast cancer by analyzing the gene expression profiles obtained from fine-needle aspirations of breast cancer[78]. From their list, we may be able to find a novel protein that is specifically stabilized in breast cancer, but degraded in normal tissues, and to apply such regulation to develop a breast cancer-targeting protein drug.

The role of apoptosis in oncogenesis is currently being studied intensively in breast cancer[79]. A decrease in the apoptosis index (AI) due to the overexpression of IAPs and the mutation of the p53 gene must lead to the resistance of cancer cells to current anticancer treatments, such as chemotherapy and radiotherapy[44]. Several studies have analyzed the prognostic significance of AI in breast carcinomas. Lipponen *et al.* showed a significant difference in survival from breast carcinoma (n = 288) depending on the AI value (cut-off point for AI was 10/mm^2)[80, 81]. Zhang *et al.* also reported a 30% difference in survival at 5 years ($p < 0.001$) in 126 patients with breast carcinoma (cut-off point for AI was 11/mm^2)[82]. These reports indicate that the induction of apoptosis prolongs the survival of patients with breast carcinoma. In this sense, a protein, which has the potential to induce cellular apoptosis, may be a reliable candidate to be introduced into the breast carcinoma.

Chromosome 3P allele loss is a frequent event in a variety of common sporadic cancers, and breast carcinoma is no exception. To analyze the extent and frequency of 3p allelic losses in early stage invasive sporadic breast carcinoma, loss of heterozygosity analysis was carried out using a 3p microsatellite marker by Martinez *et al.*[83]. They reported that 6 out of 22 tumors showed loss at 3p25-24, including the von hippel landau locus. In such breast carcinomas, IGF-IR signaling must not be suppressed because of the VHL-deficiency, resulting in unrestricted cell proliferation. Therefore, the introduction of VHL$_{104\text{-}123}$ with TAT-PTD is expected to show an antitumor effect. Moreover, VHL-deficiency must lead to the stabilization of HIF-1α protein in such breast carcinomas. Since the stability of TOP3 is regulated by pVHL via the same ODD-regulation as the HIF-1α protein, TOP3 must be stabilized even in the aerobic regions of such breast cancers, as well as in the hypoxic regions. Therefore, we can expect addi-

tional efficacy of TOP3 toward this type of breast cancer.

The accessibility of breast carcinomas permits the use of a polarographic needle electrode to measure the oxygen tension directly in cancer patients. Such studies have shown a significantly lower median oxygen partial pressure (pO_2) in malignant tumors compared with benign tumors and normal breast tissue. The median pO_2 values in malignant tumors, in benign tumors and in normal tissues were in the range of 23-28 mmHg, 42 mmHg and 54-65 mmHg, respectively[84, 85]. Of all readings in breast cancers, 30-40% fell below 10 mmHg, which is uncommon in normal tissue. Forty % of breast carcinomas contain almost anoxic regions in the range 0-2.5 mmHg, in which tumor cells still survive[84]. In such a microenvironment, the expression of the HIF-1α protein is usually induced. Bos *et al.* reported that HIF-1α proteins were indeed accumulated in breast cancers, and furthermore, the frequency of HIF-1α-positive cells increased in parallel with the increasing pathological stage of each sample[86]. Therefore, the novel hypoxia-targeting protein drug, TOP3, will likely show antitumor effects on malignant breast cancer.

Distribution of chemotherapeutic agents from tumor blood vessels to hypoxic tumor cells is also limited and thus only poor efficacy is usually obtained in conventional cancer chemotherapy. On the other hand, biologically active proteins could be delivered into whole tumor including hypoxic tumor cells, after i.p. injection with the fusion protein genetically conjugated with TAT-PTD[16]. This result demonstrates that TAT-PTD solves the problem of the poor delivery of anticancer agents to hypoxic tumor cells. Therefore, we can expect further effects of TOP3 on hypoxic cells in breast cancer.

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RONBON 2

論 文

低酸素を標的とした生体イメージング分子プローブの開発

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はじめに

三大死因である「がん」「心疾患」「脳血管疾患」の全死因における割合は、1935年には17.6%だったものが1970年には54.7%、1990年には61.6%に急増している[1]。これらの疾患に共通するキーワードが『低酸素』である。生体内に潜む異常な低酸素状態を確実に、感度良くイメージするプローブを開発することができれば、上記三大疾患の早期発見を可能にし、早期治療や、新規治療法の開発に貢献できる。心筋梗塞・脳梗塞などの虚血性疾患における『低酸素』は、広く知られている。梗塞部位周辺の組織細胞が低酸素状態にあるうちに発見し、治療することができれば、死亡率を減らすことができると共に、予後を大幅に改善できる。がんにおける『低酸素』は、あまり知られていないが、固形がんには低酸素領域が存在し（図1）、悪性度の高いがんでより多く含まれていることが臨床で報告されている。また、1mm以下の微小ながんにも、低酸素領域が存在することが、動物実験で示されており、初期のがんや転移がんの早期発見のための良い標的になる。特定の臓器がんに特徴的な分子を標的にする「分子標的」は、特定のがんを治療する上では、非常に有効で、臨床上も大きな成果をあげつつある。一方で、どこにできるかわからない「がん」を早期に発見・治療するためには、がんに共通して存在し、かつ正常組織には存在しない特徴を標的にする必要がある。固形がんに共通して存在する『低酸素』は、正常組織には存在しないため、最適な標的と考えられる。もう一歩進めて、人間ドックのような集団検診で『低酸素』を調べることができるようなイメージングプローブを開発できれば、上記三大疾病を同時に検査することが可能になるかもしれない。我々は、そのようなイメージングプローブの開発をめざし、低酸

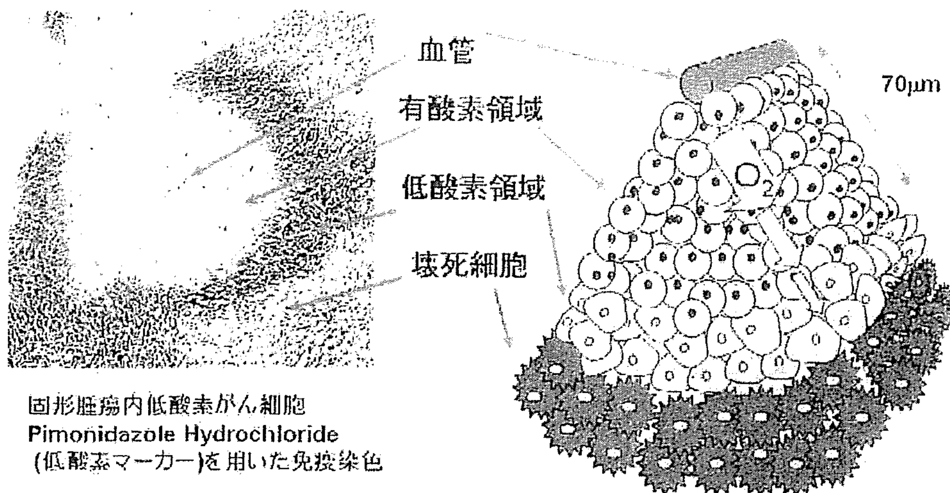


図1 固形腫瘍内低酸素領域

左は、腫瘍切片を低酸素マーカーpimonidazoleを用いて免疫染色し、腫瘍内低酸素領域(茶色)を染めた。右は、腫瘍の構造を模式的に示した。血管からの距離が大きくなるにつれて、酸素や栄養濃度が低くなり、pimonidazoleで認識されるような低酸素状態となり、それを越えると酸素も栄養も枯渇して細胞は死んでしまう。

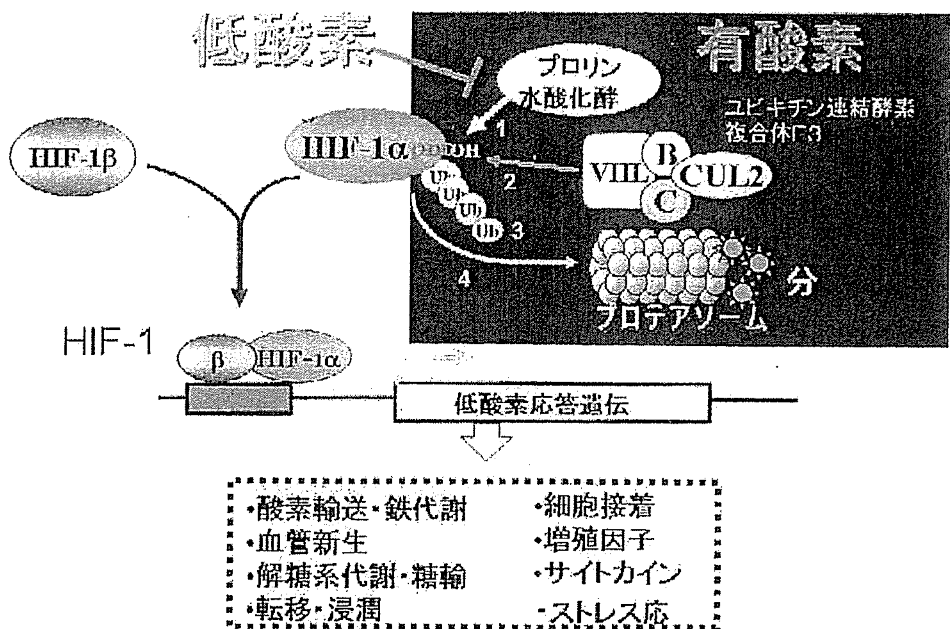


図2 HIF-1 αの酸素依存的安定性制御

HIF-1タンパク質は、HIF-1αとHIF-1 βの二量体からなる転写因子で、低酸素応答遺伝子の転写制御領域にあるHRE(hypoxia responsive element)に結合して、図(下)に示している様な様々な機能をもつ遺伝子の転写を誘導する。HIF-1 αは、低酸素状態の細胞内では安定して存在する(左上)が、有酸素状態の細胞では図内の1~4の反応によって速やかに分解される(右上)。1. プロリン水酸化酵素によりODDドメイン内のプロリン残基が水酸化される。2. その修飾をユビキチン連結酵素複合体E3が認識して、VHLを介して結合し、3. HIF-1 αはユビキチン化される。4. ユビキチン化されたHIF-1 αは、プロテアソームに運ばれ分解される。

素に特異的に安定化する融合蛋白質を応用したイメージングプローブを構築している。

酸素濃度依存的なタンパク質安定性制御機構

低酸素状態にある細胞には、極めて興味深いタンパク質が存在する。そのタンパク質はHIF-1 αと呼ばれ、転写因子HIF-1を構成する2つのサブユニットのひとつで、低酸素環境で安定化し、通常の酸素環境下(有酸素環境)で速やかに分解される[2]。すなわち、HIF-1は低酸素環境下で特異的に安定化して、転写因子として機能し、一連の低酸素応答遺伝子の発現を誘導する(図2)。それらの遺伝子は、低酸素細胞が過酷な環境に順応するために必要な因子やがんの悪性化に関与する因子をコードしており、現在までに40以上の遺伝子が報告されている[3]。

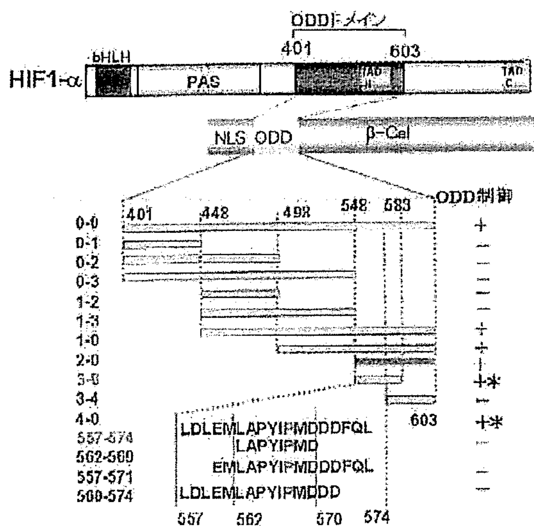
我々は、HIF-1 αタンパク質の酸素濃度依存的分解(ODD:oxygen-dependent degradation)機構に着目した。この制御機構は、2001年にプロリン水酸化酵素がクローニングされることによって、ほぼ全貌が解明された[4]。即ち、有酸素状態では、プロリン水酸化酵素が、HIF-1 α

タンパク質の中央付近にあるODDドメインのプロリン残基を水酸化し、これを目印にして結合するユビキチン連結酵素複合体E3により、HIF-1αはユビキチン化されプロテオソームに運ばれて分解される（図2）。このプロリン水酸化酵素が機能する際に、酸素と鉄を必要とする。低酸素状態では、これらが欠如するためプロリン水酸化酵素が機能せず、HIF-1αは分解されない。このODD制御は極めて厳密で、低酸素下で安定化したHIF-1αは、有酸素にすると数分以内に分解される。この極めて厳密なODD制御機構を応用することから、我々の低酸素特異的イメージングプローブの構築が始まった。

PTD-ODD 融合タンパク質の構築

我々がまず行ったことは、上記のHIF-1αにあるODDドメインを任意のタンパク質に融合させることで、任意のタンパク質が持つ機能を酸素濃度依存的に制御することができるか否かの

図3 ODD融合タンパク質のODD制御領域の同定



HIF-1α ODDドメインの一部とβ galactosidaseタンパク質の融合タンパク質をコードした遺伝子を構築した。それらを細胞に導入し，有酸素と低酸素状態で培養後β-galactosidase活性を比較した。低酸素状態のβ-galactosidase活性が有酸素状態よりも優位に高い融合タンパク質を(+)、+よりややODD制御が弱い場合を(+*)、制御がみられない場合(−)と判定した。

検証であった。ODDドメイン全部（約200アミノ酸）を付加すると、全体の分子量がかなり大きくなるので、最小のアミノ酸配列を決定するためにODDドメインの一部を含んだ一連のODDドメイン変異体を構築し、β-ガラクトシダーゼに融合させて、β-ガラクトシダーゼ活性の酸素濃度依存性を調べた（図3）。その結果、少なくとも18個のアミノ酸があれば、任意のタンパク質の活性を酸素依存的に制御できること、最適なODD制御のためには、約50個のアミノ酸配列からなるODDドメインが必要であることがわかった[5]。

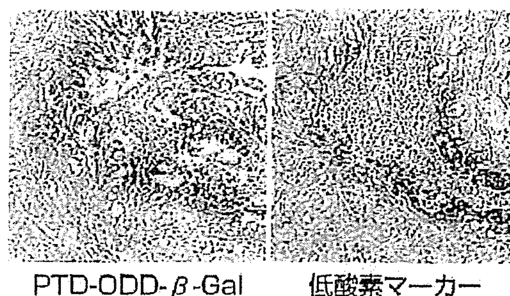
ODD制御できるタンパク質が作れても、細胞内に導入できなければ、細胞内で行われるODD制御を受けることができない。そこで我々は、タンパク質に膜透過性を付加する膜透過ドメイン（PTD）をODD融合タンパク質に付加することにより、培養細胞を用いた実験では、ほぼ100%の細胞にタンパク質を導入し、酸素依存的に機能させることに成功した。しかもこのPTDを融合させたタンパク質は、ネズミを用いた実験で、腹腔内に投与すると、脳を含む全身の組織細胞にデリバリーできることが示されている[6]。そこで我々は、PTD-ODD-β-ガラクトシダーゼを腹腔内に投与し、この融合タンパク質の体内分布とβ-ガラクトシダーゼ活性を調べた。その結果、ODDを付加していないタンパク質を投与した場合、正常肝組織と腫瘍組織全体で、タンパク質と活性が確認できたのに対し、ODDを付加したタンパク質を投与した場合は、正常組織ではタンパク質も活性も確認できず、腫瘍組織でも一部でのみタンパク質と活性が確認できた。つまり、PTD-ODD融合タンパク質は、正常組織や腫瘍の大部分の酸素が十分にある状態（有酸素状態）では分解され、腫瘍の低酸素領域でのみ安定化することを示唆していた。その事を確認するために、低酸素マーカーとし

て知られている化合物（pimonidazole）を用いて腫瘍切片を染色したところ、低酸素マーカーと同一のところに、β-ガラクトシダーゼタンパク質が存在していることがわかった（図4）。これらの結果は、我々の設計通り、PTD-ODD融合タンパク質が、全身投与で低酸素細胞がある組織までデリバリーされ、酸素濃度依存的制御を受け、低酸素細胞特異的に分布・機能することを示している。つまり、β-ガラクトシダーゼの代わりに可視化できる分子をPTD-ODD融合タンパク質とうまく組み合わせることにより、低酸素細胞特異的イメージングが可能になることを示している。

イメージングプローブの開発

現在、PTD-ODD融合タンパク質を用いたイメージングプローブの開発は、PET、MRIによるイメージングに対応できる様に研究を進めているが、基礎的データが容易に集められることから、現在機能ドメインに光イメージングに対応する物を融合させ、マウスを用いた光イメージングを行っている。より特異性の高いPTD-ODD融合タンパク質にするための最適化実験を行うと同時に、PTD-ODD融合タンパク質の有効性の検証を行っている。光イメージングプローブの開発は、まずPTD-ODD融合タンパク質に蛍光タンパク質、EGFP（蛍光波長510nm）やRed2（蛍光波長582nm）を融合させることから始めた。蛍光タンパク質は、種類が豊富で、多重染色が可能なことから、研究の幅を広げてくれるが、光の透過性が極めて悪いことから、小動物の場合でも可視化は極めて難しい。透過性を高めるためには、なるべく波長の長い光を応用することが必要になる。より波長の長い蛍光化合物Cy5.5（蛍光波長670nm）、Cy7（蛍光波長

図4 PTD-ODD融合タンパク質と低酸素マーカーの局在比較



PTD-ODD-β-galactosidaseタンパク質を担がんマウスの腹腔内に投与し、4時間後に腫瘍を切除して連続切片を作製した。切片を抗β-galactosidase抗体（左）と低酸素マーカー（右）を用いて免疫染色し、染色パタンを比較した。

767nm）で融合タンパク質を修飾し、データの収集を行っている。

現在、蛍光の退光時間を自動的に算出することにより、ほぼ正確に蛍光を発している細胞の深さや大きさを3次元で画像化するソフトを搭載しているイメージング機器もあり、将来的には多種多様にある蛍光タンパク質を使い分けて、多重染色法を用いて、ひとつの腫瘍から複数の情報を同時に得ることが可能になると考えられる。個体レベルでがん細胞を画像化する方法としては、PETやMRIが主流であるが、少なくともマウスに於いては、光イメージングが簡便性と経済性において、PETやMRIよりも優れており、多彩なプローブを同時に使うことが可能なことから、得られる情報量もPETやMRIをしのぐものになると思われる。ただし、光プローブには透過性の限界があり、大型動物やヒトへの応用は現時点では難しい。光プローブそのものの革新的進歩が無い限り、臨床への応用は、体表面に近いがんの診断や術中利用に限定されると思われる。

低酸素低酸素がん細胞の可視化

プローブの低酸素特異性を生体イメージングで検証するためには、まず標的をイメージング

する必要がある。我々は既に、がんの低酸素領域を可視化することに成功している[7]。上述した低酸素特異的転写因子HIF-1は、低酸素条件下で活性化されるので、その活性をモニターすることにより、腫瘍内の低酸素がん細胞を可視化することができる。具体的には、低酸素特異的転写因子HIF-1の結合塩基配列（hypoxia responsive element；HRE）を含む低酸素応答プロモーターの制御下に蛍光タンパク質GFPや、化学発光酵素ルシフェラーゼの遺伝子を繋いだレポーターベクターを構築し、これを安定に組み込んだがん細胞株を樹立する。この細胞を移植して形成した腫瘍の低酸素がん細胞では、低酸素応答プロモーターからのレポーター遺伝子の発現が誘導され、レポータータンパク質の蓄積が起こる。我々は、HREを5個タンデムに繋いだ配列を持つ低酸素応答プロモーター5HREの下流にルシフェラーゼを繋いだプラズミドp5HRE-luciferaseを組み込んだヒトがん細胞を樹立した（図5のA）。これをヌードマウスに移植すると、形成した腫瘍内の低酸素環境に応答してルシフェラーゼタンパク質が発現され（図5のB）基質であるルシフェリンを投与後一定期間、発光反応を起こす。この化学発光を、冷却高感度CCDカメラを搭載したイメージング機器in vivo imaging system（IVIS）を用いて可視化することができる（図5のC）。つまり、このシステムを用いることにより、同一担がんマウスの固形腫瘍内低酸素領域の変化を定量的に、リアルタイムで、何度でも経時的に観察することが可能である。

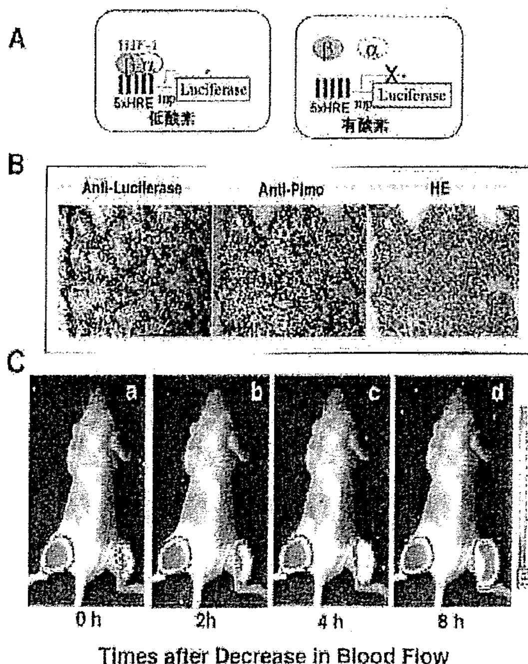
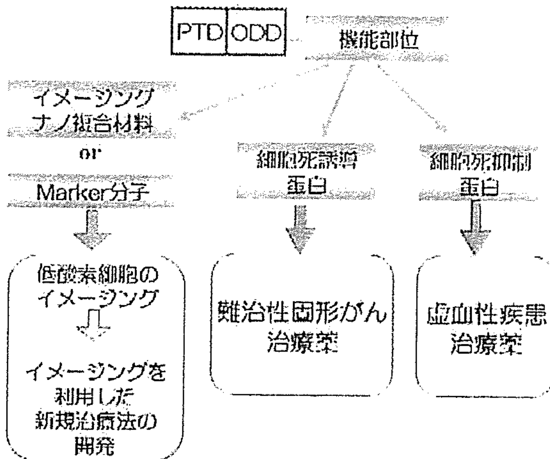


図5　ルシフェラーゼによる化学発光を用いた低酸素がん細胞の可視化
A. 5HRE-luciferaseレポータープラズミドを組み込んだヒトがん細胞。低酸素状態の細胞では、転写因子HIF-1がHRE配列に結合してルシフェラーゼが発現する（左）が、有酸素状態の細胞では、HIF-1αが分解されるためHIF-1が形成されずルシフェラーゼの発現は起こらない（右）。
B. Aで示したがん細胞をヌードマウスに移植して形成した固形腫瘍の連続切片を抗ルシフェラーゼ抗体（左）、抗低酸素マーカーPimonidazole抗体（中央）で免疫染色した。生細胞（紫色）と死細胞（桃色）を識別するためにHE染色（右）を行ったところ、ルシフェラーゼの発現している領域が低酸素マーカーで染色された領域（ともに濃い茶色）が一致しており、ルシフェラーゼの発現領域が低酸素がん細胞であることが確認できた。
C. Aのがん細胞を移植して作った固形腫瘍がある右足の付け根を紐でしばって血流を下げることで、腫瘍内の低酸素領域を人工的に増やし、ルシフェラーゼの発現を経時的（結搾直後、2、4、8時間後）に調べた。左足の腫瘍は、イメージング操作の誤差を防ぐためのコントロールで、左足のイメージが実験を通して一定になるように画像処理をした。

今後の課題

このように、標的となる低酸素の可視化は、ルシフェラーゼを用いたレポーターシステムにより達成された。標的となる低酸素特異的プローブはPTD-ODD融合タンパク質を光、PET、MRIに対応する修飾を行うことで、より臨床に近いプローブを構築するための研究が進んでいる。既に光プローブは、標的であるがんの低酸素領域に分布することを示唆する結果を予備実験で得ている。低酸素特異的光イメージングプローブの完成は間近い。PTD-ODD融合タンパク質を用いたプロジェクト（図6）は、イメージングのみに留まらず、機能ドメインに細胞死誘導機能を持つタンパク質に代えることで、抗がん作用を持たせることができる[8) 9)]。また、細胞死を抑制する機能があるタンパク質に代える



PTD-ODD融合タンパク質は、細胞膜透過ドメイン(PTD)、低酸素制御ドメイン(ODD)と機能ドメインの3つからなる。PTDは生体内でのデリバリー機能をもち、低酸素領域に融合タンパク質を効率よく運ぶ。ODDは低酸素特異性を融合タンパク質に付与する。機能ドメインには、低酸素特異的に機能させたいタンパク質をつけることができる。イメージングのための機能を付けることにより、低酸素特異的イメージングプローブをつくることができる(左)。細胞死誘導機能を付けることにより、低酸素がん細胞特異的抗がん剤を開発できる(中央)。細胞死抑制機能をつけることにより、虚血性疾患の治療薬を開発することができる(右)。

ことで、虚血性疾患の治療薬としての開発も可能である。つまり、機能ドメインを色々と代えることにより、ターゲティングが可能であり、その研究も進んでいる。

おわりに

我が国では、がんの治療を受け250万人のうち、5年以上15年以内の生存者は約100万人と推定されている。即ち、がんの治療を終え、治った約100万人が、その後再発と転移の不安をかかえながら、長い人生を歩む時代にあるといえる。定期的なモニターによるがんの早期発見は、このようなハイリスク群のみならず、通常の検診でがんの早期発見を望んでいる予備群に対しても重要であることは言うまでもない。更に、心筋梗塞、脳梗塞、動脈硬化症といった虚血性疾患を初期段階で発見できるようなプローブを開発できる可能性も十分ある。今、我が国は世界のどの国も経験したことのない速度で人口の高齢化が進行中であり、三大疾病の患者の急増も予想されている。早期発見・早期治療が重要であることは言うまでもない。しかし現在の画像診断では、基本的に正常組織との違いを探して『異常』を見つけ出すという手法が取られるため、画像診断の教育を受けた専門医の知識と経験に大きく依存している。それでは、とても急増する需要に追いつくことができない。そのためにも誰が見ても明らかに『異常』の存在を示唆できるような特異的な画像を提供できるプローブの開発が必須である。つまり、「異常が無ければ画面にはなにも写らず、イメージとして画面に映し出されたら、そこに異常がある」という極めて簡単な画像診断を提供でき、三大疾患のいずれでも早期に検出が可能であるプローブ、そんな夢のようなイメージングプローブの開発をめざしている。

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7 蛍光の生体イメージングへの応用

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Key words：光透過性，自家蛍光，近赤外波長，インドシアニングリーン，Quantum dots，GFP

Abstract
*In vivo*蛍光イメージングは、PETやMRI、CTと比較して，低侵襲，簡便，安価，迅速，プローブの多様性という点で優れている。基礎研究分野ではマウスでの全身的なイメージングが行われ，蛍光標識物質の体内動態や腫瘍など病態解明への応用が盛んである。一方臨床分野での蛍光応用は，低透過性や自家蛍光の問題から，術中や表層病変のイメージングに限定されている。現在，自家蛍光の影響を抑えより高い透過性を目指して近赤外蛍光物質の開発が盛んに行われており，臨床応用に向けての研究が注目を集めている。

はじめに

光イメージングはPET，MR，CTと並び*in vivo*イメージングの主要な分野であり，基礎研究を中心に発展している。近年，化学発光を用いた小動物の個体レベルでのイメージング報告が急増しており，蛍光イメージングの個体レベルへの応用にも期待が集まっている。近赤外領域に蛍光波長をもつ蛍光物質を利用することで，蛍光標識プローブの生体内動態が観察可能になりつつあることから，*in vivo*蛍光イメージングもその特性を生かした実用化研究の段階に入ったといえる。本稿では*in vivo*蛍光イメージングの現在について具体例を挙げつつ概説する。また基礎および臨床分野での応用例を紹介する。

1．*In vivo*蛍光イメージングの特徴

*In vivo*蛍光イメージングとは，内在性あるいは外来性の蛍光物質に対し外部から励起光を照射し，生じた蛍光を検出する方法で，標的物質の挙動をモデル生物が生きたままの状態で観察することができる。光を利用する利点として①放射線被爆などを伴わず非侵襲（低侵襲）であり，同一個体に繰り返し処置を行うことが可能である②測定設備が他に比べ安価である③原理が簡易であり観測に高度な技術を要しない④観測結果が迅速に得られる（数秒～数分）⑤遺伝子発現の情報が得られるなどが挙げられる。さらに蛍光イメージングでは蛍光物質の種類が豊富であるため，複数の標識化プローブを用いて，同一個体から複数の生体内情報を同時に収集することが可能である。また，蛍光イメージング分野ではこれまでに*in vitro*研究での膨大なデータが

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