Antitumor Effect of TAT-Oxygen-dependent Degradation-Caspase-3 Fusion Protein Specifically Stabilized and Activated in Hypoxic Tumor Cells

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ABSTRACT

Human solid tumors contain hypoxic regions that have considerably lower oxygen tension than normal tissues. These impart resistance to radiotherapy and anticancer chemotherapy, as well as predisposing to increased tumor metastases. To develop a potentially therapeutic protein drug highly specific for solid tumors, we constructed fusion proteins selectively stabilized in hypoxic tumor cells. A model fusion protein, oxygen-dependent degradation (ODD)-β-galactosidase (β-Gal), composed of a part of the ODD domain of hypoxia-inducible factor-1α fused to β-Gal, showed increased stability in cultured cells under a hypoxia-mimic condition. When ODD-β-Gal was further fused to the HIV-TAT protein transduction domain (TAT47–57) and i.p. injected to a tumor-bearing mouse, the biologically active fusion protein was specifically stabilized in solid tumors but was hardly detected in the normal tissue. Furthermore, when wild-type (WT) caspase-3 (Casp3WT) or its catalytically inactive mutant was fused to TAT-ODD and i.p. injected to a tumor-bearing mouse, the size of tumors was reduced by the administration of TAT-ODD-Casp3WT but not by TAT-ODD-mutant Casp3. TAT-ODD-Casp3WT did not cause any obvious side effects on tumor-bearing mice, suggesting specific stabilization and activation of the fusion protein in the hypoxic tumor cells. These results suggest that the combination of protein therapy using a cytoxic TAT-ODD fusion protein with radiotherapy and chemotherapy may provide a new strategy for annihilating solid tumors.

INTRODUCTION

Solid tumors comprise >90% of all of human malignancies. Furthermore, biologically and therapeutically significant hypoxia occurs in many solid tumor masses. The hypoxic tumor cells are more resistant to radiotherapy and chemotherapy than their well-oxygenated counterparts and are capable of stimulating angiogenesis by the release of several growth factors (e.g., vascular endothelial growth factor, angiogenin, and platelet-derived growth factor) for endothelial cells (1–3). Although the specific delivery of anticancer gene products has been established as an effective technique has not been established.

The transcription factor, HIF-1α, is an important mediator of the hypoxic response of tumor cells and controls the up-regulation of a number of factors vital for solid tumor expansion, including angiogenic factors, such as vascular endothelial growth factor (10). The up-regulation of HIF-1α by decreased cellular O2 concentration involves increased mRNA expression, protein stabilization, nuclear localization, and trans-activation (11). Perhaps the most striking regulation occurs exclusively at the level of protein expression via changes in the half-life of the HIF-1α protein in response to hypoxia both in cultured cells and in vivo (12–14).

The ODD domain of HIF-1α is located in its central region and consists of ~200 amino acid residues. The ODD domain controls the degradation of HIF-1α by the ubiquitin-proteasome pathway, and the deletion of this entire region is required for DNA binding and transactivation in the absence of hypoxic signaling (15). Recently, the product of von Hippel-Lindau tumor suppressor gene was reported to mediate ubiquitination and proteasomal degradation of HIF-1α under normoxic conditions by interaction with the core of the ODD domain of HIF-1α (16–18).

HIV-1 TAT has been used to deliver functional biomolecules into cells. Although the entire protein can be used for this purpose, it is more efficient to use the highly basic region containing residues 49–57. Through covalent attachment to TAT47–57, several proteins have been delivered into cells, including an inhibitor of human papillomavirus type 16 (19), ovalbumin into the MHC class I pathway (20), the cyclin-dependent kinase inhibitors p27Kip1 (21) and p16INK4a (22), and a Casp3 protein (23). TAT47–57 has also been successfully used in vivo to deliver β-Gal into all tissues of the mouse, including the brain (24).

Casp3 is a major executioner protease in known apoptotic pathways. Casp3 remains dormant until the initiator caspases activate it by direct proteolysis. Activated Casp3 cleaves the inhibitor of caspase-activated DNase, resulting in cell death (25–27). Activation of Casp3 under hypoxic conditions has been reported in several cell types, such as ventricular myocytes, endothelial cells, and squamous cell lung carcinomas (28–31).

Here we demonstrated that part of the ODD domain conferred oxygen-dependent stability to a fusion protein and that the covalent attachment of TAT47–57 and ODD548–603 to bioactive β-Gal allowed quick delivery and specific stabilization in a solid tumor. Furthermore, the replacement of β-Gal with Casp3 reduced tumor masses without any obvious side effects, demonstrating the feasibility and efficacy of protein therapy using a TAT-ODD fusion protein for solid tumors.

MATERIALS AND METHODS

Cell Cultures and Reagents. The human lung adenocarcinoma cell line A549, the human embryo kidney cell line HEK293, the human pancreatic cancer cell line CF/PAC-1, and the mouse fibroblast cell line NIH/3T3 were obtained from American Type Culture Collection. A549, HEK293, and NIH/3T3 were maintained in 5% FCS-DMEM, and CF/PAC-1 was maintained in 10% FCS-Iscove’s modified Dulbecco’s medium (Life Technologies, Inc., Rockville, MD) supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml). Cbz-LLL (Peptide Institute, Inc., Osaka, Japan) was dissolved in DMSO to 10 mM. Pino hydrochloride (Hypoxyprobe-1; Natural Pharmacica International, Inc., Research Triangle Park, NC) was dissolved in normal saline (10 mg/ml).

DNA Constructs. To construct pCH/ODD plasmids encoding a series of ODD-β-Gal fusion proteins, pCH was first constructed by annealing the oligonucleotides containing Kozak and NLS sequences (5’-AGCTTGGCAT- GGGCCCTTGAAGAAGAAGAAGA-3’ and 5’-GATCTTCTTCGTC- TTCTAGGGCCATGTC-3’) and inserting the annealed DNA fragment between HindIII and BgIII sites of the pCH110 vector (Amersham Pharmacia Biotech, Milwaukee, WI). Then pDNA fragments encoding various lengths of the ODD domain of human HIF-1α were inserted between BgIII and KpnI sites.
of pCH. The cDNA fragments encoding ODD were amplified by PCR using corresponding oligonucleotides as primers from cDNA prepared from total RNA from A549. The ODD fragments in pCH/557-574, pCH/562-569, pCH/575-571, and pCH/560-574 were prepared by annealing the corresponding oligonucleotides.

To construct pBAD/PC, pBAD/3-0, and pBAD/557-574, which encode the β-Gal protein fused to TAT, TAT-ODD-3-0, and TAT-ODD557-574 respectively, pCH/TAT/PC, pCH/TAT/3-0, and pCH/TAT/557-574 were first constructed. To prepare pCH/TAT/3-0, pCH/TAT/557-574, or pCH/TAT/PC, oligonucleotides encoding TAT were inserted into the Bgl II site of pCH/3-0 and pCH/557-574 or substituted for Bgl II-KpnI ODD3-0 fragment of pCH/3-0, respectively. Then, pCH/TAT/3-0, pCH/TAT/557-574, and pCH/TAT/PC were digested with HindIII, blunted with klenow-fragment, and further digested with Smal. The blunt-Smal DNA fragments were inserted into blunt BamHI (413) and SacI sites of pBAD/His/LacZ (Invitrogen, Carlsbad, CA), resulting in pBAD/3-0, pBAD/557-574, and pBAD/PC. To construct pGEX/3-0/Cas3WT encoding TAT-ODD-3-0-Casp3, Casp3 cDNA was amplified by PCR using corresponding oligonucleotides as primers from cDNA prepared from total RNA from A549 and substituted for β-Gal cDNA in pCH/TAT/3-0, and a DNA fragment encoding TAT-ODD-3-0/Cas3WT was inserted to Smal and SacI sites of pGEX-6P-3 (Amersham Pharmacia Biotech, Uppsala, Sweden). Casp3Mt encoding a mutant Casp3(C163M) was constructed as described previously (23) and then substituted for Casp3 in pGEX/3-0/Cas3WT.

DNA Transfection, β-Gal Assay, and X-gal Staining. For β-Gal assays, cells (1 × 10^6/well) were seeded onto 24-well plates and cotransfected with 2 μg of pCH plasmids (Fig. 1, A and B) and 0.2 μg of pRL-CMV (Promega, Madison, WI) as described (32). The following day, the cells were trypsinized, and aliquots were plated in duplicate onto 24-well plates (one well without Cbz-L-LLL and the other with 5 μM Cbz-L-LLL). Later (24 h), the cells were lysed with 200 μl of 1 × Reporter Lysis Buffer (Promega). The lysates (50 μl) were subjected to the calorimetric enzyme assay in triplicate using a β-Gal Enzyme Assay System (Promega), and obtained A605nm was normalized by luminescence counts (10 s) of 10-μl lysates assayed with a Luciferase Assay System (Promega) and rabbit anti-β-Gal Ab (ICN Pharmaceuticals, Inc., Aurora, OH) by the indirect immunoperoxidase methods as described previously (33). X-gal staining was performed on tissues fixed in 4% formaldehyde in PBS and then embedded in paraffin. Sections (5 μm) were deparaffinized and rehydrated. The sections were preincubated with 1% H2O2 in PBS for 15 min. The sections were then incubated with 0.5% X-gal in PBS containing 0.1% Triton X-100 and 2 mM MgCl2 at 37°C for 4 h in the presence of the solvent (DMSO) or 5 mM Cbz-L-LLL. Then, TAT-ODD-Casp3 proteins (7.5 μg/30 μl) were added to the fresh culture medium, and the cells were further incubated for 24 h under normoxic (20% O2) or hypoxic (1% O2) conditions. The photos were taken under an inverted microscope.

Assay for Apoptosis. NIH/3T3 cells (1 × 10^5/well) were seeded onto 24-well plates and preincubated for 24 h in the presence of the solvent (DMSO) or 5 mM Cbz-L-LLL. Then, TAT-ODD-Casp3 proteins (7.5 μg/30 μl) were added to the fresh culture medium, and the cells were further incubated for 24 h under normoxic (20% O2) or hypoxic (1% O2) conditions. The photos were taken under an inverted microscope.

Assay for Casp3 Activity. TAT-ODD-Casp3 proteins (7.5 μg/30 μl) were added to the culture of HEK293 cells (5 × 10^3 Cells/0.5 ml) and incubated for 4 h in the presence of the solvent (DMSO) or 10 μM Cbz-L-LLL. The cells were harvested, and Casp3 activity in a 50-μg cell lysate was detected as the amount of chromophore p-nitroanilide after cleavage from the labeled substrate DEVD-p-nitroanilide (CPP32/Casp3 colorimetric protease assay kit; MBL, Nagoya, Japan) and quantified using a microtiter plate reader at 405 nm.

RESULTS

Determination of the Core of the ODD Domain Responsible for ODD of an ODD-Fusion Protein. Because the stability of HIF-1α is regulated through the ODD domain, we determined the stability of a protein fused to an appropriate region of ODD domain would be under the same control as HIF-1α. To identify the region responsible for ODD of an ODD-fusion protein, we constructed a series of fusion proteins composed of parts of the ODD domain of HIF-1α and β-Gal as a bioactive reporter. The plasmids encoding the NLS and various lengths of the ODD domain fused to the NLS of terminal of β-Gal (Fig. 1, A and B) were transfected into HEK293 cells. The β-Gal activity of these cells was examined by X-gal staining after culturing in the presence or absence of Cbz-L-LLL, a peptide aldehyde that inhibits the major peptidase activities of proteasomes and reduces the degradation of proteins (35). Therefore, culture with Cbz-L-LLL renders the cells in a hypoxia-mimic condition (36). The results of X-gal staining were
summarized as the ODD regulation, which refers to the increased β-Gal activity and increased number and thickness of blue color in X-gal-stained cells in Fig. 1B. In the cells transfected with the plasmids encoding fusion proteins consisting of β-Gal and the HIF-1α residues 401–447 (pCH/0-1), 401–497 (pCH/0-2), 401–547 (pCH/0-3), 448–497 (pCH/1-2), 448–547 (pCH/1-3), or 579–603 (pCH/4-0), the X-Gal staining pattern was same between the Cbz-LLL-treated and untreated cells (Figs. 1B and 2A). On the other hand, in the cells transfected with the plasmids encoding the fusion proteins consisting of β-Gal and the HIF-1α residues 401–603 (pCH/0-0), 448–603 (pCH/1-0), 498–603 (pCH/2-0), 548–603 (pCH/3-0), or 560–583 (pCH/3-4), more cells were stained deeper with X-gal in the Cbz-LLL-treated cells than the untreated ones (Figs. 1B and 2A).

To determine the core of ODD region responsible for ODD of the ODD-fusion proteins, we further narrowed down the ODD region. The residues 548–583 have 18 amino acid sequences (557–574) conserved among the related HIFs and between species. We constructed a plasmid encoding the fusion protein consisting of β-Gal and the HIF-1α residues 557–574 (ODD557–574) and examined its ODD regulation as above. Although a considerable number of cells was stained with X-gal in the absence of Cbz-LLL, more cells were stained deeper in the Cbz-LLL-treated cells than the untreated ones (Fig. 2A), suggesting that ODD557–574 still retained the ODD function. However, the stability of fusion proteins containing the HIF-1α residues of 562–596 (pCH/562–569), 557–571 (pCH/557–571), or 560–574 (pCH/560–574) were no longer under the control of oxygen tension (Fig. 1B), indicating that ODD557–574 contained the minimum ODD residues necessary for the ODD regulation of an ODD-fusion protein.

Furthermore, to examine the importance of the NLS for ODD regulation, we constructed pCH/557–574 Δ NLS and pCH/3-0 Δ NLS, which had the NLS sequence deleted from their vector constructs (Fig. 1A), and examined the stability of fusion proteins with β-Gal assay after culturing their transfectants in the presence or absence of Cbz-LLL. The ODD regulation was significantly decreased in the NLS-less fusion proteins (Fig. 2B), confirming the importance of the nuclear localization of proteins for the ubiquitin/proteasome-mediated degradation through the ODD domain of HIF-1α. For additional study, we used the fusion proteins containing NLS and HIF-1α residues 548–603 (ODD3-0) or ODD557–574.

Construction of TAT-ODD Fusion Protein and Its Oxygen-dependent Instability in Vitro. To apply the ODD-fusion proteins to in vitro study, we fused TAT77–575 to the NH2-terminal of ODD-β-Gal and created a TAT-ODD-β-Gal triple fusion protein; proteins without TAT transmembrane sequences are not delivered to any tissues (24). We first examined its ODD regulation in vitro. When the control TAT-β-Gal fusion protein was added to the culture medium of A549 cells, 100% of cells were stained by X-gal, irrespective of the oxygen condition (Fig. 2C, a and c), indicating that TAT-β-Gal fusion protein was efficiently delivered and stably localized in the cells. The addition of 10 times more β-Gal protein alone to the culture medium resulted in no staining of the cells (data not shown). When the optimal amount of TAT-ODD/3-0-β-Gal was added to the culture medium of A549 cells, the cells were stained significantly milder with X-Gal under normoxic condition than the cells under hypoxic condition (Fig. 2C, b and d). Similar results were obtained by the experiment using TAT-ODD557–574-β-Gal (data not shown). However, the degradation of the fusion protein under normoxic condition (20% O2) or in the absence of Cbz-LLL was less efficient in the experiment using the pCH/557–574 plasmid (Fig. 2, A and B). Therefore, we used TAT-ODD3-0-β-Gal for in vitro study.

Selective Stabilization of TAT-ODD-β-Gal in the Hypoxic Regions of the Solid Tumor. Next, we applied TAT-ODD/3-0-β-Gal to in vivo study to examine if it was delivered to tumors and specifically stabilized in the hypoxic regions of the tumors. Mice bearing tumors resulting from xenografts of a human pancreatic cancer cell line CF/PAC-1 were i.p. injected with a control protein, TAT-β-Gal, and the tumors were subsequently analyzed for the delivery and stability of the control protein with X-Gal staining and anti-β-Gal Ab. Entire tumors were stained with both X-Gal and anti-β-Gal Ab, indicating that bioactive β-Gal was delivered efficiently (Fig. 3A, a and c). However, when TAT-ODD/3-0-β-Gal was used for the same experiment, only parts of tumors were stained with X-Gal and anti-β-Gal
We have presented a novel protein therapy targeting solid tumors. To design a protein drug, we used the TAT protein transduction domain, which quickly and efficiently delivered a bioactive protein to all tissues and took advantage of the selective stabilization of the ODD-fusion protein under hypoxic conditions. The TAT-β-Gal fusion protein was delivered successfully in vivo into all tissues of the mouse (Ref. 24; Figs. 3A, a and c; and 4, a, c, and e), whereas TAT-ODD-β-Gal was detected in only a part of the solid tumors (Fig. 3A, b and d). To examine if the regions where TAT-ODD-β-Gal was detected were hypoxic, we compared them with the regions detected with Ab against a hypoxia marker (anti-Pimo Ab). The regions were similar, although not identical, and anti-β-Gal Ab was detected in an apparently larger area than the regions detected with anti-Pimo Ab (Fig. 3B). We speculated that the regions surrounding the hypoxic regions in the tumor would have a relatively low oxygen tension and would therefore have slower degradation of TAT-ODD/3-0-β-Gal.

**DISCUSSION**

ANTITUMOR EFFECT OF TAT-ODD FUSION PROTEIN

To design a protein drug, we used the TAT protein transduction domain, which quickly and efficiently delivered a bioactive protein to all tissues and took advantage of the selective stabilization of the ODD-fusion protein under hypoxic conditions. The TAT-β-Gal fusion protein was delivered successfully in vivo into all tissues of the mouse (Ref. 24; Figs. 3A, a and c; and 4, a, c, and e), whereas TAT-ODD-β-Gal was detected in only a part of the solid tumors (Fig. 3A, b and d). To examine if the regions where TAT-ODD-β-Gal was detected were hypoxic, we compared them with the regions detected with Ab against a hypoxia marker (anti-Pimo Ab). The regions were similar, although not identical, and anti-β-Gal Ab was detected in an apparently larger area than the regions detected with anti-Pimo Ab (Fig. 3B). We speculated that the regions surrounding the hypoxic regions in the tumor would have a relatively low oxygen tension and would therefore have slower degradation of TAT-ODD/3-0-β-Gal.

**Fig. 3. Selective stabilization of TAT-ODD-β-Gal in solid tumors.** In A, the enzymatic activity and quantification of β-Gal protein in the tumor sections were assessed by X-gal staining (a and b) and anti-β-Gal Ab (c and d) after i.p. injection of TAT-β-Gal (a and c) or TAT-ODD/3-0-β-Gal (b and d) fusion proteins. In B, Pimo was i.p. injected 2 h after i.p. injection of fusion proteins. The samples were analyzed with anti-Pimo Ab (right) or anti-β-Gal Ab (left). The Ab-stained samples (Au and Ac; B) were also stained with hematoxylin. Bar, 100 μm.

**Fig. 4. Rapid degradation of TAT-ODD-β-Gal fusion protein in normal tissue.** Analysis of β-Gal protein and enzymatic activity was assessed by anti-β-Gal Ab (a–d) and X-gal staining (e and f), respectively, in the liver. The livers were removed 4 h after i.p. injection of TAT-β-Gal (a, c, and e) or TAT-ODD/3-0-β-Gal (b, d, and f) fusion proteins to tumor-bearing mice and then stained with X-gal for 3 days. c and d, the higher magnification of a and b, respectively. The sections for X-gal staining (e and f) were not stained with hematoxylin. Bar, 100 μm.
Because the hypoxic regions with the lowest oxygen tension would be just a few percentage of the total tumor mass, removal of the hypoxic regions alone would not influence the tumor mass significantly. The administration of TAT-ODD/3-0-Casp3\textsuperscript{WT}, however, not only suppressed the tumor growth but also reduced the tumor masses (Fig. 5C), suggesting that TAT-ODD/3-0-Casp3\textsuperscript{WT} must have stabilized in the hypoxic regions, as well as their outskirts, in a distribution similar to TAT-ODD/3-0-\(\beta\)-Gal.

When applying a cytotoxic TAT-ODD fusion protein as a protein drug, we must minimize the damage to normal tissues. To achieve this, at least three things have to be considered for additional study: (a) dosage; (b) administration; and (c) characteristics of the cytotoxic protein fused to TAT-ODD. Addressing the first point, determination of the optimal dose of the fusion protein is the crucial part of this protein therapy. Because the degradation of ODD-fusion proteins depends on the cellular ubiquitin/proteasome system, their degradation rate is restricted by the capacity of the cellular ubiquitin/proteasome system. In vitro experiments using TAT-ODD-\(\beta\)-Gal fusion protein indicated that the ODD regulation was evident at the optimal concentration of the ODD-fusion proteins, but at higher concentrations, the ODD regulation was hardly observed (data not shown). The fusion of an additional ODD domain to the COOH-terminal end of the protein might increase the efficiency of the ODD degradation. Addressing the second point, although we gave only one injection of the TAT-ODD fusion protein to the tumor-bearing mice, repeated administrations might be effective in reducing the tumor mass. The timing of the administration and the combination with radiotherapy and chemotherapy constitute the next phase of this study. Lastly, to minimize the side effects, the cytotoxic activity should preferably be indirect, and a certain level of accumulation of proteins should be needed to achieve cytotoxicity. We used a dormant form of Casp3, a nuclear executioner caspase, as a cytotoxic protein fused to TAT-ODD. We expected that Casp3 would be active in solid tumor cells under hypoxic conditions, because Casp3 activation has been observed under hypoxic conditions in vivo as well as in vitro (Refs. 28–31 and Fig. 5AB). It should be emphasized that although TAT-ODD-Casp3 was able to induce apoptosis in mouse cells under hypoxic condition (Fig. 5A), 20 mg/kg TAT-ODD/3-0-Casp3\textsuperscript{WT} did not cause any obvious side effects in the tumor-bearing mice, consistent with the data that \(\beta\)-Gal activity and protein were hardly detected in the normal tissue (Fig. 4). Although we successfully demonstrated the antitumor effect of TAT-ODD-Casp3, there might be better cytotoxic protein candidates. Therefore, it is imperative to conduct an additional study.

We determined the minimum ODD residues (557–574) that can regulate the ODD of a fusion protein. This region is also located within the minimal NH\textsubscript{2}-terminal trans-activation domain (532–585; Refs. 38 and 39), which has been identified recently as the region critical for interaction with von Hippel-Lindau to mediate the ubiquitin-proteasomal degradation of HIF-1\(\alpha\) under normoxic conditions (16–18). The ODD residues required for the ODD regulation of HIF-1\(\alpha\) stability (36) were different from the ones required for the regulation of ODD-fusion proteins (Fig. 1B). Srinivas et al. (40) obtained similar results using a Gal4/hHIF-1\(\alpha\) (530–652) fusion protein. At present, we are unable to explain these differences. The ODD regulation of HIF-1\(\alpha\) \textit{per se} might need an additional regulatory factor. Because the ODD regulation of ODD/3-0 was tighter than those of ODD/557–574 and ODD/548–583, there might be some regulatory element within the region 584–603. The molecular mechanism of the ODD regulation in ODD-fusion proteins is under investigation.

Fig. 5. The antitumor effect of TAT-ODD-Casp3. In A, NIH/3T3 cells were treated with WT or Mut TAT-ODD-Casp3 proteins or the buffer and cultured for 24 h under normoxic (20% \(O_2\)) or hypoxic (1% \(O_2\)) conditions. In B, WT or Mut TAT-ODD-Casp3 proteins or the buffer were added to the culture of HEK293 cells and incubated for 4 h in the presence of the solvent only (dark bars) or Cbz-LLL (gray bars). The cells were harvested, and Casp3 activity in a 50-\(\mu\)g cell lysate was measured and shown in the figure. C, tumor-bearing mice were i.p. injected with the buffer only (0 mg/kg) or 2 or 20 mg/kg TAT-ODD/3-0-Casp3 fusion proteins (Mut or WT). Average total volume of the tumors was \(\approx 300 \text{ mm}^3\). The size of tumors was measured every day after injection, and the relative tumor mass compared with the mass on the injected day was indicated in the figure. Tumors were measured with calipers, and tumor volume was calculated as \(0.5 \times L \times W^2\). The results are the mean of six independent tumors \(\pm SD\).