Targeted Nanoparticles That Deliver a Sustained, Specific Release of Paclitaxel to Irradiated Tumors

Ralph J. Passarella1, Daniel E. Spratt1, Alice E. van der Ende2, John G. Phillips1, Hongmei Wu1, Vasanth Sathiyakumar2, Li Zhou1, Dennis E. Hallahan3,4, Eva Harth2, and Roberto Diaz5,6

Abstract

To capitalize on the response of tumor cells to XRT, we developed a controlled-release nanoparticle drug delivery system using a targeting peptide that recognizes a radiation-induced cell surface receptor. Phage display biopanning identified Gly-Ile-Arg-Leu-Arg-Gly (GIRLRG) as a peptide that selectively recognizes tumors responding to XRT. Membrane protein extracts of irradiated glioma cells identified glucose-regulated protein GRP78 as the receptor target for GIRLRG. Antibodies to GRP78 blocked the binding of GIRLRG in vitro and in vivo. Conjugation of GIRLRG to a sustained-release nanoparticle drug delivery system yielded increased paclitaxel concentration and apoptosis in irradiated breast carcinomas for up to 3 weeks. Compared with controls, a single administration of the GIRLRG-targeted nanoparticle drug delivery system delayed the in vivo tumor tripling time by 55 days ($P = 0.0001$) in MDA-MB-231 and 12 days in GL261 ($P < 0.005$). This targeting agent combines a novel recombinant peptide with a paclitaxel-encapsulating nanoparticle that specifically targets irradiated tumors, increasing apoptosis and tumor growth delay in a manner superior to known chemotherapy approaches. Cancer Res; 70(11); 4550–9. ©2010 AACR.

Introduction

In vitro, many agents are capable of killing cancer cells effectively. These agents trigger cancer cell death through numerous complex pathways, such as apoptosis or prevention of further cell division. However, when these agents are transferred from use in cell culture to an entire system, the effect on normal tissue limits their use in a clinical setting. With a small therapeutic index between cancer destruction and toxic side effects, drugs are often not used in patients or discontinued far before they achieve a maximal effect.

Thus, targeted therapy provides a means to circumvent the toxicities and lack of treatment response of conventional systemic chemotherapy. With the development of targeted biologicals, such as trastuzumab and imatinib, therapies for specific cancer types have been developed. However, these therapeutics are often limited to cancers expressing various mutations and thus are limited in broad use (1, 2). Treatments aimed at universal solid tumor therapy, such as angiogenesis inhibitors, have had limited success thus far (3).

The discovery of receptors expressed at much greater levels on tumors than on normal tissue would provide targets for drug delivery. Therefore, receptor induction in tumors could play a critical role in providing new targets. Ionizing radiation (XRT), although also therapeutic, could potentially cause cellular stress localized to cancer cells, which may cause new receptor translocation. Beyond its cytotoxic effects, XRT has been shown to induce gene transcription (4) and protein expression on tumor microvasculature (5). Using phage display biopanning, recombinant peptides that bind only to treated cancers have been found (6, 7). However, combining these peptides with chemotherapeutic agents has not been effectively translated to clinical use.

Through targeted therapeutics, nanoparticle delivery systems have the potential to overcome the normal tissue toxicity of traditional chemotherapy. However, although paclitaxel encapsulation in albumin nanoparticles, nab-paclitaxel, increases the efficacy and safety over paclitaxel formulated in Cremophor (8–10), more nausea, diarrhea, and grade 3 sensory neuropathy occurs in patients treated with nab-paclitaxel (8). Another remaining challenge of nanoparticle delivery systems is the lack of control of drug release profiles. This distinct “burst effect,” in which the majority of drug is released in a rapid and uncontrollable fashion, creates unpredictable pharmacokinetics, thereby making effective dose regimens difficult to predict.

Our goal is to identify a novel recombinant peptide and a radiation-inducible receptor pair in XRT-treated cancers and
conjugate the peptide to a novel nanoparticle drug delivery system (DDS) for use in XRT targeted chemotherapy. In this work, we present three new findings: the discovery of a recombinant peptide that recognizes XRT-treated tumors, the discovery of the radiation-inducible receptor of this peptide, and the therapeutic benefits of a targeted recombinant peptide/nanoparticle DDS.

Materials and Methods

Animals used
Athymic nude and C57/Bl6 mice were purchased from Harlan Laboratories. All animal protocols were approved by the Institutional Animal Care and Use Committee.

Tumor models
GL261 murine glioma and MDA-MB-231 human breast cancer cell lines were purchased from American Type Culture Collection. Heterotopic tumor models were developed by s.c. inoculating cell suspensions (6 x 10⁶ cells) into nude or C57/Bl6 mice.

 Coculture assays
Human umbilical vein endothelial cells (HUVEC) in the sixth passage (Lonza) and GL261 murine glioma cells were cocultured as previously described (7). The cells were allowed to interact for 1 day before treatment with 3 Gy XRT and incubated for 3 hours after they were harvested. Coverslips were blocked for 30 minutes with 5% bovine serum albumin and 1% streptavidin (ThermoScientific). Cells were incubated for 1 hour with a streptavidin-peptide-AlexaFluor594 complex (AlexaFluor594 carboxylic acid succinimidyl ester was purchased from Invitrogen; Supplementary Fig. S1). HUVEC nuclei were stained with 4',6-diamidino-2-phenylindole, and images of nuclei and peptide binding were taken by Vanderbilt Cell Imaging Shared Resource Center using a Zeiss Axiophot fluorescent microscope at 40× magnification. Assays were performed in triplicate.

Near IR imaging
Tumor-bearing mice were treated with three once-daily doses of 3 Gy XRT or sham XRT (three per group) and injected with peptide or antibody 3 hours after the last XRT treatment. In one experiment, labeled complexes of biotinylated peptide-AlexaFluor750 conjugates were injected (AlexaFluor750 carboxylic acid succinimidyl ester was purchased from Invitrogen). In a second experiment, an antibody to 78-kDa glucose regulated protein (GRP78) conjugated with AlexaFluor750 was injected and tumors were removed 7 days after labeled antibody injection; polyclonal serum IgG antibody was used as a control. In a third experiment, mice received an unlabeled blocking antibody to GRP78 or unlabeled polyclonal IgG serum and then injected with the labeled Gly-Ile-Arg-Leu-Arg-Gly (GIRLRG) peptide. Near IR images were taken using the IVIS imaging system with an ICG filter setting at various time points after the injection.

Membrane protein extraction
GL261 tumor samples either treated with 3 Gy XRT or sham XRT were removed from the hind limbs of athymic nude mice 48 hours after treatment and frozen at −80°C. Forty-milligram samples of treated and untreated frozen tumors were homogenized, and the protein was extracted using the Mem-PER Eukaryotic Membrane Protein Extraction Kit (ThermoScientific). The extracted protein was then incubated overnight in the Slide-A-Lyzer Dialysis Cassettes (ThermoScientific). The protein was then incubated overnight with NeutrAvidin-coated agarose beads (ThermoScientific) bound to biotinylated GIRLRG or scrambled peptide (RILGGR). After incubation, the beads were washed with 1× PBS, boiled at 100°C, and run on an Invitrogen NuPAGE 10% gel. The gel was stained with Invitrogen Simply Blue SafeStain. Bands from the gel were analyzed by the Vanderbilt Proteomics Core through the liquid chromatography/tandem mass spectrometry technique.

Western blotting
Nude mice implanted s.c. in the hind limb with either GL261 and MDA-MB-231 tumors were treated with 3 Gy XRT for 3 consecutive days, and tumors were removed 48 hours post-XRT. Tumor samples were homogenized, and the protein from the samples was extracted. Protein was probed with antibodies for GRP78 and actin (Cell Signaling) on a polyvinylidene difluoride membrane and exposed to film that was later developed using the Western Lightning Chemiluminescence Plus detection system (Perkin-Elmer) according to the manufacturer’s protocol.

Immunohistochemistry
Paraffin-embedded tumor samples were stained using an antibody for the von Willebrand factor (vWF; DakoCytomation) at a 1:100 dilution from the original stock solution of 3.1 g/L and incubated overnight. Samples were then incubated with a streptavidin-peptide-AlexaFluor594 complex and washed three times with PBS.

In a second assay, samples were stained with vWF and incubated with an antibody to GRP78 at dilutions of 1:250 and 1:1,000 of original stock solution. These samples were then incubated with streptavidin-peptide-AlexaFluor594 complex and imaged.

Images were taken using a fluorescent microscope at 20× magnification. Assays were performed in triplicate.

Nanoparticle synthesis and attachment of GIRLRG peptide to nanoparticles
Polyester nanoparticle DDS was synthesized by the procedure described by van der Ende and colleagues (11).
KKCGGGGIRLRG peptide (56 mg, 3.35 μmol) in dimethylsulfoxide (DMSO; 2 mL) was added to a solution of nanoparticles from poly(valerolactoneepoxyvalerolactone-allylvalerolactone-oxepanedione) containing 11% epoxide and cross-linked with 1 equivalent of 2,2-(ethylenedioxy)bis(ethylamine) per epoxide (ref. 11; 105.6 mg, 0.78 μmol) in DMSO (1 mL). The reaction mixture was heated for 72 hours at 34°C. Residual peptide was removed by dialyzing with SnakeSkin Pleated Dialysis Tubing (molecular weight cutoff 10,000) against 50/50 THF/CH3CN.

Figure 1. GIRLRG binds to tumors responding to XRT. A, biopanning of the T7 phage–based peptide library followed by sequencing was used in vivo to identify phage peptides binding specifically to glioma (GL261) treated with 3 Gy XRT and 40 mg/kg sunitinib. Shown here are the three amino acid sequences isolated most frequently and the percentage of peptide-bearing phages in the sequenced clones. B, GIRLRG preferentially binds to radiation-treated cocultures of GL261 gliomas and HUVECs. GIRLRG (top row) or a scrambled peptide (middle row) was incubated on HUVECs from XRT-treated and untreated GL261/HUVEC cocultures. GIRLRG was also incubated on XRT-treated and untreated HUVEC cultures alone (bottom row). DAPI, 4′,6-diamidino-2-phenylindole. C and D, GIRLRG binds to tumors responding to XRT ex vivo and in vivo. C, nude mice were implanted in the right hind limb with GL261 glioma cells and were either left untreated or treated daily with radiation (3 Gy) for 5 consecutive days (three mice per treatment group). Tumor sections were incubated with vWF, an endothelial marker, followed by fluorescently labeled GIRLRG, and imaged. Untreated tumor sections and XRT-treated tumor sections 48 hours posttreatment are shown. D, nude mice were implanted in the hind limb with GL261 glioma cells and were either left untreated or treated daily with radiation (3 Gy) for 3 consecutive days (three mice per treatment group). Fluorescently labeled GIRLRG peptide preferentially binds to radiation-treated tumors in GL261 xenografts compared with untreated tumors.

Encapsulation of paclitaxel in GIRLRG-conjugated nanoparticles

Paclitaxel was encapsulated by the procedure described by van der Ende and colleagues (12). The weight percent of paclitaxel encapsulated in the nanoparticles was determined by NanoDrop UV-Vis at 254 nm as mentioned in the literature and was found to be 11.3%.

Paclitaxel antibody staining

Nude mice were implanted in the hind limb with MDA-MB-231 tumors. Once tumors reached 450 mm³ in volume, mice were treated with 3 Gy XRT once daily for 3 consecutive days or were left untreated. On the 2nd day, mice were injected with one of either (a) systemic paclitaxel, (b) paclitaxel/nanoparticle with RILGGR, or (c) paclitaxel/nanoparticle with GIRLRG. The paclitaxel concentration used was 10 mg/kg. Tumors were removed 1 and 3 weeks after treatment, embedded in paraffin, and sectioned. Tumor sections were incubated with a monoclonal antibody to paclitaxel (Santa Cruz Biotechnology) at a concentration of 1:500. Three mice per group were used. All paclitaxel antibody staining was performed in triplicate.
Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining

Nude mice were treated and tumors were collected as described in the above section. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was done with the DeadEnd Colorimetric TUNEL System (Promega) following the manufacturer’s instructions. Positive staining was observed by light microscopy.

Paclitaxel antibody staining and TUNEL staining evaluation

All slides were evaluated and graded based on color intensity of immunoreactions using a six-tier grading system of 5 to 6 (strong), 3 to 4 (moderate), 1 to 2 (faint), and 0 (none). Assays were performed in triplicate.

Statistical analyses

Student’s t test was used to perform group comparisons. Linear correlations of peptide binding and tumor response to treatment were developed by using the correlation coefficient of tumor growth and radiance data sets (SigmaPlot).

Results

Discovery of a peptide that recognizes irradiated tumors

We used phage display technology to identify a targeting peptide that would discriminately bind to irradiated tumors. Using a previously characterized in vivo biopanning method to screen the T7 phage–based random peptide library (6, 7), the novel peptide GIRLRG was identified as the predominant phage-encoded peptide recovered from irradiated GL261 gliomas in mice (Fig. 1A). We then investigated the specificity of GIRLRG for irradiated tumors using in vitro coculture experiments. To model the tumor microenvironment, we used HUVECs cocultured with GL261 tumor cells. These experiments revealed that the GIRLRG targeting peptide bound to tumor vasculature only when two criteria were met: tumor cells were irradiated and tumor cells were able to interact with HUVECs (Fig. 1B). To simulate normal tissue, HUVECs were cultured alone. There was no binding of the GIRLRG recombinant peptide to this normal tissue model, suggesting an obligate interaction between the tumor and tumor vasculature for the target receptor of the peptide to be available for binding. We also found that the GIRLRG peptide colocalizes with an endothelial cell marker in irradiated tumor samples ex vivo (Fig. 1C). We then used an in vivo binding model using near IR imaging with GL261 tumors implanted in the hind limbs of mice. The GIRLRG targeting peptide was again shown to preferentially bind to radiation-treated tumors in vivo over untreated tumors (Fig. 1D).

GRP78 is induced by XRT in tumors

We next sought to discover the target of GIRLRG. Agarose beads coated with GIRLRG were incubated with membrane

Figure 2. GRP78 is induced by XRT. A, the highlighted sequences are the sequences identified by mass spectrometry as extracted and precipitated with GIRLRG peptide coated on agarose beads. B, GRP78 is induced in HUVECs grown in coculture with GL261 gliomas after XRT treatment. GL261 glioma cells (3 × 10⁵) and HUVECs (1 × 10⁴) were cocultured for 24 hours before treatment with 3 Gy XRT. Three hours posttreatment, AlexaFluor594-labeled GRP78 antibodies were added to the culture plates. C, Western blot of GRP78 expression in XRT-treated and untreated (No Tx) GL261 glioma and MDA-MB-231 breast carcinoma tumor sections showing that GRP78 is upregulated to the membrane in response to XRT. D, antibody to GRP78 binds selectively to XRT-treated tumors. GL261 tumors were implanted in the hind limbs of nude mice and treated with 3 Gy XRT for 3 consecutive days. On the 3rd day, an antibody to GRP78 or IgG antibody serum conjugated with AlexaFluor750 was injected through the tail veins of mice. Tumors were removed 7 days after peptide injection and imaged using near IR imaging to assess relative levels of peptide binding.
protein extracts from irradiated GL261 tumors and removed 48 hours postirradiation. We isolated a 78-kDa band by gel electrophoresis. Mass spectrometry of that band revealed it to be GRP78 (Fig. 2A). Therefore, we investigated the effects of radiation on GRP78 concentration.

In vitro, we found that GRP78 is induced in HUVECs grown in coculture with GL261 gliomas after XRT treatment (Fig. 2B). Membrane protein extract from GL261 gliomas and MDA-MB-231 breast carcinomas were analyzed for GRP78 expression through Western blot (Fig. 2C). The results revealed increased GRP78 expression after radiation treatment, in both tumor types, compared with controls. GRP78 upregulation in response to XRT in GL261 gliomas was validated ex vivo (Supplementary Fig. S2) and in vivo (Fig. 2D). Tumors implanted in the hind limbs of nude mice were treated with XRT, then injected with either a polyclonal antibody to GRP78 or IgG antibody serum. Three hours after antibody administration, mice were injected with GIRLRG peptide labeled with AlexaFluor750. C, mice were imaged 24 hours after the final XRT treatment using near IR imaging. D, graph of relative peptide binding in vivo in mice treated with XRT and injected with either an antibody to GRP78 or an IgG control antibody serum. Differential peptide binding is shown as the percentage of tumor fluorescence increase relative to an untreated tumor-bearing mouse injected with an IgG control antibody serum.

**Figure 3.** GRP78 is the target of GIRLRG. A, GL261 glioma cells and HUVECs were cocultured for 24 hours before treatment with 3 Gy XRT followed by blocking antibody to GRP78 or a control IgG antibody serum. Cells were then incubated with AlexaFluor594-labeled GIRLRG peptide and imaged for peptide binding. B, GL261 gliomas implanted in the hind limbs of nude mice were treated with 3 Gy XRT daily for 3 days, sectioned and incubated with a polyclonal antibody to GRP78 at antibody concentrations of 1:1,000 and 1:250. C and D, GL261 gliomas implanted in the hind limbs of nude mice (three mice per treatment group) were treated with 3 Gy XRT daily for 3 days. One hour after the final XRT treatment, mice were injected with either a polyclonal antibody to GRP78 or IgG antibody serum. Three hours after antibody administration, mice were injected with GIRLRG peptide labeled with AlexaFluor750. C, mice were imaged 24 hours after the final XRT treatment using near IR imaging. D, graph of relative peptide binding in vivo in mice treated with XRT and injected with either an antibody to GRP78 or an IgG control antibody serum. Differential peptide binding is shown as the percentage of tumor fluorescence increase relative to an untreated tumor-bearing mouse injected with an IgG control antibody serum.

**GRP78 and GIRLRG Interaction Studies**

We next studied the putative ligand-receptor interaction between the GIRLRG peptide and GRP78. This was accomplished through in vitro, ex vivo, and in vivo experiments using blocking antibodies to GRP78. In vitro cocultures with GL261/HUVECs showed decreased binding of GIRLRG to HUVECs when blocking antibodies to GRP78 were added to the coculture (Fig. 3A). Next, we treated implanted GL261 tumors in mice with XRT, removed and sectioned the tumors, and treated with varying concentrations of GRP78 blocking antibody. We found that as the GRP78 blocking antibody concentration increased, binding of fluorescently labeled GIRLRG decreased (Fig. 3B). An in vivo imaging study was performed to assess if fluorescently labeled GIRLRG peptide could still bind to XRT-treated tumors following the addition of an antibody to GRP78. The GRP78 antibody attenuated GIRLRG signal intensity by >70% compared with control IgG serum antibody in irradiated GL261 tumors (P < 0.05; Fig. 3C and D).

**Creation of a GIRLRG-Targeted Nanoparticle DDS**

We postulated that conjugating the GIRLRG recombinant peptide with a nanoparticle DDS could target chemotherapeutics specifically to radiated tumors. After nanoparticle formation (11), the peptide was conjugated using a high-yielding thiol-ene reaction, reacting the free thiol of the cysteine near the NH2 terminus of the KKCGGGGIRLRG with allyl functionalities on the nanoparticle (12). Nuclear magnetic resonance...
spectroscopy methods could determine the conjugation of 37 peptides (Fig. 4). In the final step, paclitaxel was incorporated, resulting in a DDS that is well dispersed in a Cremophor-free solution. The biocompatibility of peptide-targeted particles in concentrations applied for in vivo studies was confirmed in cytotoxicity assays (Supplementary Fig. S3).

GIRLRG-targeted nanoparticle DDS increases paclitaxel concentration and apoptosis in irradiated tumors

We next investigated the effects of the GIRLRG-targeted nanoparticle DDS on paclitaxel concentration and apoptosis in tumors compared with controls. MDA-MB-231 breast carcinomas were implanted in the hind limbs of nude mice and treated as described in Fig. 5. Tumors were harvested at 1 and 3 weeks posttreatment, and the levels of paclitaxel (Fig. 5A and B) and apoptosis (Fig. 5C and D) were determined with the respective cell staining assays and quantified. Paclitaxel was found in significantly greater concentrations in the targeted nanoparticle group with the use of irradiation over all other treatment groups at 1 and 3 weeks \((P < 0.05; \text{Fig. } 5\text{A and } B)\). Similarly, TUNEL staining of these tumor sections showed that at 1 and 3 weeks, the nanoparticle-GIRLRG DDS was superior to radiation and systemic paclitaxel in maintaining persistent cytotoxicity \((P < 0.05; \text{Fig. } 5\text{C and } D)\). In fact, staining for paclitaxel and apoptosis significantly persisted for 3 weeks after just a single administration of the nanoparticle over the other control groups \((P < 0.05)\), indicating that the nanoparticle-GIRLRG peptide complex provides a prolonged and sustained release of paclitaxel when properly targeted to the tumor with XRT.

Treatment with targeted nanoparticle DDS produces in vivo tumor growth delay

Our primary outcome to determine the overall efficacy of our novel targeting nanoparticle DDS was to assess tumor volume tripling time in human tumor cell lines and in syngeneic mouse tumors. Therefore, we implanted MDA-MB-231 breast carcinomas in nude mice and GL261 gliomas in C57/B16 mice and performed a tumor growth delay study after treating the mice as shown in Fig. 6. Our results showed that MDA-MB-231 tumor tripling time was delayed 55 days with the nanoparticle-targeted peptide with XRT \((P = 0.0001)\), compared with 11 to 14 days by the three other XRT treatment groups \((P < 0.05; \text{Fig. } 6\text{A})\). Both unirradiated nanoparticle groups provided no significant tumor growth delay when...
compared with the untreated control, suggesting that even nanoparticle-GIRLRG is not adequately targeted in unirradiated tumors. The administration of radiation with systemic paclitaxel or with untargeted nanoparticle (nanoparticle-RILGGR) provided no significant tumor growth delay when compared with radiation alone (Fig. 6A). Similarly, in the GL261 group, tumor tripling time was significantly delayed by 12 days by nanoparticle-targeted peptide with XRT treatment ($P < 0.005$); however, all other treatment groups failed to significantly delay tumor tripling time compared with untreated controls (Fig. 6B).

**Discussion**

We began designing our targeted DDS by seeking peptides capable of recognizing irradiated cancer cells. Using a previously characterized *in vivo* biopanning method (6, 7, 13, 14), we discovered several candidate peptides (Fig. 1A). One of the candidates, GIRLRG, proved to be specific to irradiated cancer cells capable of interacting with tumor vascular endothelial cells *in vitro* (Fig. 1B) and tumors *ex vivo* and *in vivo* (Fig. 1C and D). For the GIRLRG peptide to be of clinical utility for targeted therapy, its receptor must not be a ubiquitously expressed surface protein. XRT provides an intense cellular stress, causing activation of DNA damage repair cascades and endoplasmic reticulum stress pathways (15). Therefore, we hypothesized that the receptor for GIRLRG could potentially be involved in one of these stress pathways and be induced by XRT.

We sought to find this receptor using GL261 tumor membrane protein affinity purification with GIRLRG. The receptor identified for GIRLRG is GRP78 (Fig. 2A).
known as an endoplasmic reticulum chaperone involved in suppression of stress-induced apoptosis (16) but can exist as a cell surface protein to transduce extracellular stimuli to intracellular signals to promote tumorigenesis (17–19). Signaling through cell surface GRP78 increases cytosolic calcium concentration, Akt phosphorylation, IP3, and NF-κB, leading to an increase in DNA and protein synthesis as well as cellular proliferation (19). A breadth of research supports the correlation of GRP78 to higher pathologic grade, metastasis, chemotherapeutic response, cancer prognosis, and patient survival in gliomas and breast carcinomas (16, 18, 20–23). Importantly for clinical translation, GRP78 is expressed at much higher levels in a variety of tumors and tumor vasculature compared with much lower levels in normal tissues and non–tumor-bearing vasculature where expression potentially increases during tissue inflammation (17, 18, 20, 23–25). This “natural” gradient of GRP78 expression between tumor vasculature and non–tumor-bearing vasculature is consistent with our in vitro data of GIRLRG selectively binding only to irradiated HUVECs incubated with cancer cells, not to HUVECs alone (Fig. 1B).

The discovery that GRP78 is upregulated at the cell surface in XRT-treated tumors and tumor vasculature (Fig. 2) may be a further indicator of its role in the cellular stress response, and in the ability of a cancer cell to escape stressors that would lead a normal cell to apoptosis (16, 20–23). The mechanism by which GRP78 translocates to the cell surface is not fully understood, but hypotheses include particular mechanisms adapted by cancer cells, oversaturation of the endoplasmic reticulum retention system, transmembrane cycling of endoplasmic reticulum GRP78 to the cell surface, and cotrafficking with cell surface client proteins (21). Because GRP78 is expressed at the cell surface of tumors but not...
normal organs, cell surface GRP78 has become an attractive target for targeted therapy (21). Ligand peptides for GRP78 are rapidly internalized through clathrin-mediated endocytosis (26). Previously, GRP78 targeting peptides linked with paclitaxel (27, 28), doxorubicin (28), or proapoptotic peptides (26) have been shown to induce melanoma cell death in vitro. Once weekly systemic administration over 4 weeks of proapoptotic chimeric peptides fused to GRP78 binding motifs suppressed tumor growth in xenograft models without affecting normal organs (17). Nonetheless, these observations have not been approved for clinical use.

Having discovered a receptor induced by XRT, we sought to use nanoparticle technology to deliver a paclitaxel drug payload using GIRLRG as a targeting molecule for GRP78. We postulated that conjugating the GIRLRG recombinant peptide with a nanoparticle DDS capable of controlled pharmacokinetics could target chemotherapeutics specifically to radiated tumors. In addition, the control over particle sizes has been recognized to be crucial to predict the interaction with cells and other biological barriers (29) and reduce the risk of undesired clearance from the body through the liver or spleen (30). Therefore, the nanoparticle we used applies an intermolecular cross-linking technique (31) that not only allows for predetermined nanoparticle dimensions with SDs of 10% but also provides adjustable cross-linking densities to control the degradation of the particles and allows for postmodification reactions with bioactive groups such as the targeting peptide. The adjustable cross-linking densities of the nanoparticle-targeting peptide complex can be applied toward the controlled and sustained release of paclitaxel. Consistent with the nanoparticle biodegradation profile (12, 31), paclitaxel was found in significantly greater concentrations in our MDA-MB-231 breast cancer xenografted mice in the targeted nanoparticle group with the use of XRT over all other treatment groups at 1 and 3 weeks (P < 0.05; Fig. 5A and B). Similarly, TUNEL staining for apoptosis at both 1 and 3 weeks were greatly increased, indicating that the nanoparticle-GIRLRG DDS was superior to radiation and systemic paclitaxel in maintaining persistent cytotoxicity (P < 0.05; Fig. 5C and D). Our data support the model that the GIRLRG peptide is able to achieve significant targeting of paclitaxel to the tumor (Fig. 5A and B) when there is high expression of GRP78 at the surface, which can be induced in tumors with XRT (Fig. 2).

Our primary outcome to determine the overall efficacy of our novel targeting nanoparticle DDS was to assess tumor volume tripling time in both human tumor cell lines and in syngeneic mouse tumors. Our results showed that MDA-MB-231 tumor tripling time was delayed 55 days with the nanoparticle-targeted peptide with XRT (P = 0.0001), compared with 11 to 14 days by the three other XRT treatment groups (P < 0.05; Fig. 6A). Both unirradiated nanoparticle groups provided no significant tumor growth delay when compared with the untreated control, suggesting that even the nanoparticle-GIRLRG complex itself is not adequately targeted in unirradiated tumors. The administration of radiation with systemic paclitaxel or with untargeted nanoparticle (nanoparticle-RILGGR) provided no significant tumor growth delay when compared with radiation alone (Fig. 6A), suggesting that the nanoparticle itself (nanoparticle-RILGGR) does not target irradiated tumors. Similarly, in the GL261 group, tumor tripling time was significantly delayed by 12 days by nanoparticle-targeted peptide with XRT treatment (P < 0.005); however, all other treatment groups failed to significantly delay tumor tripling time compared with untreated controls (Fig. 6B). Thus, a single administration of the targeted nanoparticle DDS achieved tumor growth delay in irradiated tumors that was significantly greater than conventional systemic chemotherapy and radiation.

In conclusion, our results indicate that administration of XRT to tumors and tumor vasculature causes migration of GRP78 to the cell surface where the nanoparticle-GIRLRG DDS specifically delivers paclitaxel to the radiated site. By combining the controllable, sustained drug release of the nanoparticle with the newly identified GIRLRG targeting peptide, we were able to specifically target chemotherapeutics directly to an XRT-inducible receptor causing significant tumor cell death. The receptor identified for the peptide, GRP78, is an ideal target for our nanoparticle-peptide DDS because it is inducible by XRT (Fig. 2). Even after a single administration of the nanoparticle-GIRLRG complex, paclitaxel can be detected in radiated tumors after 3 weeks, which translates into significantly increased levels of apoptosis and tumor growth delay (Figs. 5 and 6). Thus, we have used novel nanotechnology in vivo to produce a significant increase in the efficacy of cancer treatment over current clinical models. We expect our targeted nanoparticle DDS to have clinical utility, and with further investigation hope to implement our system into clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Samuel Spratt for figure illustrations; Erkki Ruoslahti (Burnham Institute) for the gift of T7 phage-based random peptide library; Vanderbilt University Medical Center Cell Imaging Shared Resource Center; Vanderbilt Academic Fund Venture Capital for Proteomics, and Vanderbilt Mass Spectrometry Core for experimental support; and Jessica Huamani and Allie Fu for technical support. Roberto Diaz is a recipient of the Leonard B. Holman Research Pathway fellowship.

Grant Support

Department of Defense Breast Cancer Research Program grant BC061828 (R. Diaz); National Science Foundation Career Award CHE-0645737 (E. Harth); start-up funds from Vanderbilt University (E. Harth) and Emory University (R. Diaz); Resident Research Seed Grant from the American Society for Radiation Oncology (R. Diaz); NIH grant R01-CA112385 (D. Hallahan); Vanderbilt In Vivo Cellular and Molecular Imaging Center grant P50CA128323 (D. Hallahan); StarBRITE microgrant from Vanderbilt University (R. Passarella, D. Spratt, and R. Diaz).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 01/28/2010; revised 03/17/2010; accepted 03/30/2010; published OnlineFirst 05/18/2010.
Radiation-Induced GRP78 Mediates Targeted Drug Delivery

References

Targeted Nanoparticles That Deliver a Sustained, Specific Release of Paclitaxel to Irradiated Tumors

Ralph J. Passarella, Daniel E. Spratt, Alice E. van der Ende, et al.

Cancer Res 2010;70:4550-4559. Published OnlineFirst May 18, 2010.

Updated version Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-10-0339

Supplementary Material Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2010/05/17/0008-5472.CAN-10-0339.DC1

Cited articles This article cites 31 articles, 11 of which you can access for free at: http://cancerres.aacrjournals.org/content/70/11/4550.full.html#ref-list-1

Citing articles This article has been cited by 9 HighWire-hosted articles. Access the articles at: /content/70/11/4550.full.html#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.