Noninvasive Radiofrequency Field Destruction of Pancreatic Adenocarcinoma Xenografts Treated with Targeted Gold Nanoparticles

Evan S. Glazer1, Cihui Zhu1, Katheryn L. Massey1, C. Shea Thompson2, Warna D. Kaluarachchi1, Amir N. Hamir3, and Steven A. Curley1,4

Abstract

Purpose: Pancreatic carcinoma is one of the deadliest cancers with few effective treatments. Gold nanoparticles (AuNP) are potentially therapeutic because of the safety demonstrated thus far and their physiochemical characteristics. We used the astounding heating rates of AuNPs in nonionizing radio-frequency (RF) radiation to investigate human pancreatic xenograft destruction in a murine model.

Experimental Design: Weekly, Panc-1 and Capan-1 human pancreatic carcinoma xenografts in immunocompromised mice were exposed to an RF field 36 hours after treatment (intraperitoneal) with cetuximab- or PAM4 antibody–conjugated AuNPs, respectively. Tumor sizes were measured weekly, whereas necrosis and cleaved caspase-3 were investigated with hematoxylin–eosin staining and immunofluorescence, respectively. In addition, AuNP internalization and cytotoxicity were investigated in vitro with confocal microscopy and flow cytometry, respectively.

Results: Panc-1 cells demonstrated increased apoptosis with decreased viability after treatment with cetuximab-conjugated AuNPs and RF field exposure ($P = 0.00005$). Differences in xenograft volumes were observed within 2 weeks of initiating therapy. Cetuximab- and PAM4-conjugated AuNPs demonstrated RF field–induced destruction of Panc-1 and Capan-1 pancreatic carcinoma xenografts after 6 weeks of weekly treatment ($P = 0.004$ and $P = 0.035$, respectively). There was no evidence of injury to murine organs. Cleaved caspase-3 and necrosis were both increased in treated tumors.

Conclusions: This study demonstrates a potentially novel cancer therapy by noninvasively inducing intracellular hyperthermia with targeted AuNPs in an RF field. While the therapy is dependent on the specificity of the targeting antibody, normal tissues were without toxicity despite systemic therapy and whole-body RF field exposure. Clin Cancer Res; 16(23); 5712–21. ©2010 AACR.
nanoparticles are candidates for hyperthermic treatment, but gold has the most immediate potential for use in human patients and seems to have a favorable safety profile (5, 16, 17).

On the basis of previous in vitro work (8), we hypothesized that systemic delivery of antibody targeted gold nanoparticles (AuNPs) would induce hyperthermic cytotoxicity after RF field exposure in human pancreatic carcinoma xenografts without injury to normal tissues. Antibodies to 2 distinct human antigens (EGFR-1 and MUC-1) were utilized to deliver 2 AuNPs of different sizes to 2 unique human pancreatic xenografts. Although EGFR-1 is a problematic therapeutic target due to its diverse constitutive expression in normal tissues, PAM4 is a human antibody to MUC-1 that is specific to pancreatic carcinoma (18). The components were chosen such that the constructs had similar sizes that could lead to increased tumor internalization rates (19). The primary aim was to show human pancreatic cancer xenograft destruction.

Translational Relevance

Nanoparticle-mediated hyperthermic therapy offers a treatment that potentially will simultaneously have less adverse effects than systemic chemotherapy and a more direct action on pancreatic cancer cells. Gold colloids have a long history of minimal adverse effects, whereas nonionizing radiation is known to be safe. However, because AuNPs heat in nonionizing radiation, there is the potential to noninvasively target or direct the hyperthermic effect. Pancreatic cancer, one of the deadliest cancers, has multiple targeted therapies in development. As we have shown here, the proper combination of a targeting antibody conjugated with AuNPs results in effective tumor destruction with minimal toxicity.

Materials and Methods

Cell culture, antibodies, fluorophores, and gold nanoparticles

Two human pancreatic carcinoma cell lines, Panc-1 and Capan-1, were acquired from the American Type Culture Collection (ATCC) confirmed by the Characterized Cell Line Core Service (M. D. Anderson Cancer Center; December 2009), and maintained according to ATCC’s cell media recommendations in standard conditions (37°C, 5% CO2). All experiments utilized standard cell culture coated dishes and equipment (BD Biosciences; Corning Inc.). Cetuximab (C225; Bristol-Myers Squibb), a chimeric monoclonal IgG1 and equipment (BD Biosciences; Corning Inc.). Cetuximab All experiments utilized standard cell culture coated dishes and equipment (BD Biosciences; Corning Inc.). Cetuximab against a mucin glycoprotein, MUC-1, was directly conjugated to 20-nm AuNPs (Ted Pella, Inc.) via a thiol–gold interaction. First, PAM4 IgG was washed in borate buffer (pH 8) twice and resuspended at a concentration of 2 mg/mL. A monovalent, single-molecule IgG (Fc + Fab with reactive sulfur groups on the heavy chain formerly of the disulfide bond) was created by reducing the interchain disulfide bond at the hinge region with 3 molar excess of tris(2-carboxyethyl)phosphine, which is a relatively gentle, but very specific, nonsulfur-containing disulfide bond reducing agent that does not reduce heavy-light intrachain disulfide bonds or internal disulfide bonds at the antigen binding site at these concentrations (21). Subsequently, 450 μg of “activated” PAM4 was mixed with 1,000 μg of 20-nm AuNP in borate buffer and mixed for 4 hours in the dark at room temperature to permit the reduced sulfur moieties to directly form thiol–gold bonds on the AuNPs. The construct was twice washed with borate buffer, concentrated 15-fold in a 50,000 molecular weight centrifugation filter unit (Millipore Corp.) at 3,800 × g.

Because PAM4 does not have the same glycosolation status in the Fc region as C225, it was directly conjugated to slightly larger AuNPs without a linker via a thiol–gold interaction. First, PAM4 IgG was washed in borate buffer (pH 8) twice and resuspended at a concentration of 2 mg/mL. A monovalent, single-molecule IgG (Fc + Fab with reactive sulfur groups on the heavy chain formerly of the disulfide bond) was created by reducing the interchain disulfide bond at the hinge region with 3 molar excess of tris(2-carboxyethyl)phosphine, which is a relatively gentle, but very specific, nonsulfur-containing disulfide bond reducing agent that does not reduce heavy-light intrachain disulfide bonds or internal disulfide bonds at the antigen binding site at these concentrations (21). Subsequently, 450 μg of “activated” PAM4 was mixed with 1,000 μg of 20-nm AuNP in borate buffer and mixed for 4 hours in the dark at room temperature to permit the reduced sulfur moieties to directly form thiol–gold bonds on the AuNPs. The construct was twice washed with borate buffer, concentrated 15-fold in a 50,000 molecular weight centrifugation filter unit (Millipore Corp.) at 3,800 × g.

A small shift in the peak plasmonic absorbance of the AuNPs (NS1; Applied NanoFluorescence) was indicative of a nonaggregated conjugation state after challenge with equivolume of 10% sodium chloride (21). Dynamic light scattering (DLS; Horiba, Ltd.) determined the average hydrodynamic diameter of the constructs (500 measurements per sample in triplicate).

Target protein expression in Panc-1 and Capan-1 cells with immunoprecipitation

The cell membrane expression of EGFR-1 and MUC-1 was confirmed by Western blot analysis. Briefly, cell pellets were lysed with cold radioimmunoprecipitation assay buffer and centrifuged at 13,000 rpm for 30 minutes. The protein extracts (30 μg per lane) were electrophoresed on Bis-Tris protein gel, transferred to a polyvinylidene difluoride membrane, and sequentially incubated in 5% dry milk and primary antibodies (BD Biosciences and Immunomedics, Inc.). Next, the membranes were incubated with secondary anti-human IgG antibodies (H + L chains; Jackson ImmunoResearch, West Grove, PA). Images were acquired by a high-resolution photoscanner (CanoScan 4400F;
Confocal imaging of antibody-conjugated AuNPs in vitro

Confocal images were prepared by growing Panc-1 cells on standard 1.5 glass cover slips, which were treated with AlexaFluor (AF) 647–labeled C225 (the primary antibody delivered to living cells), 10-nm AuNP alone, or the labeled C225-AuNP-conjugated construct, or neither for 3 hours. Cell membranes were stained with 5 µg/mL of wheat germ agglutinin conjugated to AF 594 (Invitrogen Corp.) by incubating the cover slip for 10 minutes at 37°C. After phosphate buffered saline (PBS) washings, the cells were fixed with 4% paraformaldehyde and permeabilized with cold 100% methanol. Blocking was performed for 1 hour with blocking solution [3% bovine serum albumin (BSA) + 1% animal serum in PBS]. Cells were washed and stained with AF 488–labeled secondary antibody against the heavy and light chains of human IgG (Invitrogen Corp.). After washing, DNA was stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; 300 nmol/L) for 5 minutes. Cover slips were mounted (Dako Denmark A/S, Glostrup, Denmark), dried, sealed, and stored in the dark at 4°C until analyzed (Olympus DSU with Orca II ER camera; Olympus America Inc., Center Valley, PA).

In a separate experiment, Panc-1 cells were treated with AF 647–labeled C225 at the same concentration with or without AuNPs to determine the percentage of cells in vitro with measurable amounts of primary fluorophore–labeled C225-AuNP. Cells were identified in the usual manner by an automated cytomter while the fluorescence of AF 647 was simultaneously measured for individual cells (Cellometer Vision; Nexcelom Bioscience, LLC). A fluorescence threshold of 7 arbitrary units was chosen to separate positive from negative populations, based on the control sample. For each group, 1,800 cells were counted and a histogram was created for each group with the same binning (Sigma Plot Version 11; Systat Software Inc.).

TEM imaging in vitro

Cell pellets were fixed with a 3% glutaraldehyde/2% paraformaldehyde solution in 0.1 mol/L of cacodylate buffer at pH 7.4. Samples were washed with 0.1% cacodylate-buffered tannic acid, treated with 1% buffered osmium tetroxide, and stained with 1% uranyl acetate. The samples were ethanol dehydrated and embedded in LX-112 medium. After polymerization, the samples were partially digested with 2 mL of certified 30% H2O2, evaporated. Next, 5 mL of aqua regia (1 part of nitric acid combined with 3 parts of hydrochloric acid by volume, in a fume hood) was slowly added to each sample as the temperature was slowly raised to 130°C in order to completely digest the samples. After 3 hours, the samples were passively cooled, and diluted with 18-MΩ water to a final volume of 10 mL. The gold concentration of each sample was determined by inductively coupled plasma atomic emission spectrometry (ICP) according to manufacturer’s recommendations (iCAP 6500; Thermo Fisher Scientific).

Nanoparticle heating in an RF field

Triplicate solutions of various concentrations of AuNPs alone or conjugated to antibodies were placed in the RF field in a transparent, 1.5-mL quartz cuvette directly on a copper ground plate and exposed to a high-voltage RF field at 600-W generator power for 1 minute (13.56 MHz, 10-cm air gap; ThermMed, LLC, Inc.). Temperatures were recorded every 30 seconds with an IR camera (FLIR SC 6000; FLIR Systems, Inc.).

Panc-1 in vitro RF field exposure and cytotoxicity after C225-AuNP treatment

Panc-1 cells were plated in quadruplicate at a concentration of 1 x 10^5 cells/mL in 60-mm dishes and incubated overnight. Negative controls remained untreated, whereas the others were treated with C225 alone or C225-AuNP. Separate groups (n = 4 each) underwent RF field exposure at 600 W for 10 minutes. Bulk media temperature remained between 36°C and 41°C, as measured by an IR camera (FLIR SC 6000). Viability was measured with flow cytometry (LSRII; BD Biosciences) 36 hours after exposure. Briefly, cell media (i.e., dying cells that were floating) was collected and the adherent remaining cells were released with trypsin. The trypsin was then neutralized with cell media and the cells were collected. Each sample was washed and stained without fixation or permeabilization. After a final wash, 50,000 cellular events were acquired for each sample and analyzed with FlowJo 8.8.6 (TreeStar, Inc.). After gating the single cell population, Annexin V, a protein that binds to membrane protein phosphatidylserine, positive cells were considered apoptotic; whereas propidium iodide (PI), a chemical that fluoresces when bound to DNA, positive cells were considered necrotic. Annexin V and PI double-positive cells were considered dead cells, whereas double-negative cells were characterized as viable.

Panc-1 and Capan-1 pancreatic xenografts AuNP treatment and RF field exposure

Nude balb/c mice (NCI Mouse Repository) were subcutaneously injected with 3 x 10^6 Panc-1 or Capan-1 cells to the right flank (n = 20 each cell line). After the tumors were palpable (approximately 3 weeks), mice within each cancer cell type were randomly assigned to 1 of 4 groups. The mice with Panc-1 xenografts were treated/exposed to no treatment, C225-AuNP without RF exposure, RF expos-
Nanoparticle and RF Field–Induced Tumor Destruction

sure alone, or C225-AuNP treatment plus RF exposure. The mice were treated with C225-AuNP at 10 mg/kg by gold weight injected intraperitoneally to prevent AuNP collection at the site of an extremity (i.e., tail vein). RF exposure consisted of 600-W generator power for 10 minutes with an air gap of 10 cm. All antibody-AuNP treatments occurred weekly, whereas all RF exposures occurred 36 hours after treatments on a weekly basis. Tumors were measured 48 hours after RF exposure each week, whereas C225-AuNP treatment and RF exposure began after week 1.

Mice with Capan-1 tumors were randomly assigned to 1 of 4 groups as well (n = 5 each group). Those groups were untreated control, PAM4-AuNP treatment only, unconjugated AuNP treatment plus RF field exposure (600-W generator power, 10-minute duration, and 10-cm air gap), and PAM4-AuNP treatment weekly with the same RF exposure weekly. Unconjugated AuNP and PAM4-AuNP were also treated intraperitoneally at 10 mg/kg by gold weight for each.

During the experiment, all mice were kept in accordance with an Institutional Animal Care and Use Committee approved protocol. To safely expose the animals to RF fields, their tails, ears, and paws were completely grounded to avoid excess current in the extremities that would result in electrothermal injuries. This was accomplished by placing mice directly on a large grounded copper plate and attaching conducting copper tape (3M) to the extremities and plate.

Mice were sedated with ketamine 0.1 mg/g and xylazine 0.01 mg/g intraperitoneally prior to each RF field exposure and monitored thereafter. The temperature of each mouse was continuously measured with an IR camera (FLIR SC 6000), and at no time did the temperature exceed 41.5°C. For the first minute of every RF field exposure, a cuvette with 100 μg/ml of 20-nm AuNPs was placed within 1.5 cm of the tumor. This served as the control to confirm heating of AuNPs in the RF field. After 1 minute, the RF field generator was briefly turned off (<2 seconds) to remove the cuvette, as it approached boiling temperatures soon thereafter.

Tumor volumes (width squared × length) were measured weekly with electronic calipers. After 6 weeks of treatment, the animals were euthanized and selected organs (liver, spleen, kidney, lung, and tumor) were harvested for gold biodistribution and histopathologic evaluation. For evaluation of normal organ and tumor-specific injury, specimens were prepared for histologic procedures, embedded in paraffin, and sectioned at 5 μm. The sections were stained with hematoxylin and eosin stain (H&E) and examined by light microscopy. Injury was assessed by grade (grade 1: rare <10%; grade 2: mild, 10%–20%; grade 3: moderate, 20%–50%; and grade 4: severe, >50%) by an expert in comparative mammalian pathology (A.N.H.).

Confocal immunofluorescent microscopy in vivo

Tumor sections on standard glass slides were deparaffinized by heating to 60°C for 1 hour. They were then dewaxed and rehydrated by sequential washing for ~5 minutes in xylene (3×) followed by decreasing concentrations of ethanol in water. Sections were then placed in boiling antigen retrieval buffer (citrate based, pH = 6, 0.5% Tween-20). This was heated in a microwave at boiling temperatures for an additional 1 minute. Next, the sections were microwaved in the buffer for 15 minutes at 30% power. Sections were then washed with PBS and placed in blocking solution for 1.5 hours (1% BSA and 2% fetal bovine serum in PBS). Sections were stained with an antibody to cleaved caspase-3 (Cell Signaling Technology, Danvers, MA) diluted 1:250 in blocking solution for 1 hour at room temperature. Sections were thrice washed with PBS and a secondary antibody conjugated to AF 488 was applied to each (1:300) for 1 hour at room temperature. Sections were thrice washed in PBS, stained with DAPI (300 nmol/L for 10 minutes), and subjected to a final washing in PBS. Fluorescent mounting media was applied (Dako North America, Inc.) and the sections were sealed with a 1.5 glass cover slip.

Statistical methods

Results are means ± standard errors of the mean unless otherwise noted. Statistical significance, α, was set to P = 0.05. Two-tailed Student’s t test compared differences in means between groups, whereas multiple-way analysis of variation analyzed tumor volumes (SPSS Version 16.0, SPSS Inc.). Data were plotted with Sigma Plot Version 11 (Systat Software Inc.).

Results

Characterization of antibody-conjugated AuNPs

Directional conjugation of 10-nm AuNP to cetuximab via a covalent linker was confirmed by a small shift (<10 nm) in peak plasmonic absorption (Fig. 1). The small shift in the peak and similar width are consistent with AuNP conjugation and not consistent with AuNP aggregation (20). A PAM4 hemi-antibody (single heavy- and light-chain IgG with activated sulfur moieties) was conjugated to 20-nm AuNPs via a direct thiol–gold linkage and confirmed by a small shift in peak plasmonic absorption (Fig. 1). The hydrodynamic diameter of C225-AuNP is 32.6 ± 0.7 nm while it is 36.9 ± 1.5 nm for PAM4-AuNP. Conjugated and unconjugated AuNPs were heated in a concentration-dependent fashion, whereas the C225-AuNP construct was heated significantly faster in an RF field at 600 W than unconjugated AuNPs (Fig. 1D; P < 0.0001).

Time-dependent internalization of C225-conjugated AuNPs to Panc-1 cells in vitro

Confocal microscopy demonstrated qualitatively different internalization rates after various treatment durations of C225-conjugated AuNPs compared with C225 alone (Fig. 2). Briefly, the conjugation to gold produced punctate, spherical fluorescence initially along the cellular membrane, which was then internalized into the cytoplasm (Fig. 2; Supplementary Movie S1). This indirectly suggests that AuNP constructs are transported between cells (Supplementary Movie S1). C225 alone seems to have inter-
nalized faster than antibody-conjugated AuNPs. In a separate experiment, fluorescence from a fluorophore directly attached to C225 showed significant overlap in vitro to secondary antibodies conjugated to a different fluorophore, suggesting most of the primary antibody remains intact even after internalization (Fig. 3). Nearly 90% of cells contain measurable C225-AuNPs based on direct fluorescence labeling (Fig. 3; P < 0.0001 compared with background in untreated cells). In addition, after 3 hours of treatment, there were significantly more AuNPs delivered by C225 than unconjugated AuNPs (untargeted) in vitro (2.1 pg/cell ± 0.1 pg/cell vs. 0.2 pg/cell ± 0.1 pg/cell, respectively; P = 0.005).

Apoptosis-mediated cell death after C225-AuNP treatment and RF exposure in vitro
C225-AuNPs (Fig. 4A) induced significant cell death via apoptosis 36 hours after RF field exposure, based on flow cytometric analysis (P = 0.00005 compared with untreated controls; Fig. 4B). Neither C225 treatment alone (no AuNPs) with RF exposure nor C225-AuNP treatment alone without RF induced as much cell death as the combined treatment (Fig. 4B).

Destruction of Panc-1 xenografts due to C225-AuNP treatments and RF exposure without injury to other organs
Excess electrical current on the surface of mice was grounded with copper conducting tape attached to the extremities in order to prevent nonspecific electrothermal injuries (Fig. 5A). Although no mice developed electrothermal injuries during RF field treatment, AuNPs in adjacent quartz cuvettes continued to heat rapidly, demonstrating the RF-induced thermal effect (Fig. 5B; Supplementary Fig. S1). Tumor temperatures in bulk rose above body temperatures by approximately 3 °C during
the first minute of RF exposure and remained stable there-
after (Fig. 5B; Supplementary Fig. S1).

Despite C225 conjugation, the biodistribution of AuNPs
demonstrated increased concentration in the liver and
spleen, based on ICP analysis. Delivery of the C225-AuNP
construct by intraperitoneal injection yielded tissue gold
levels of $25.1 \pm 6.4$ mg of AuNP/g of tumor, $257.6 \pm 7.5$ mg of AuNP/g of spleen, $302.7 \pm 82.3$ mg of AuNP/g of liver, $4.0 \pm 0.3$ mg of AuNP/g of lung, and $4.1 \pm 0.1$ mg of AuNP/g of kidney. No murine tissues (liver, spleen, kidneys, and lung), demonstrated any evidence of acute or chronic injury based on histopathologic analysis despite the presence of AuNPs.

Although Panc-1 control tumors had baseline evidence of central necrosis consistent with an aggressive carcinoma, the tumors from mice treated with C225-AuNP + RF field exposure showed at least 1-grade increase in necrosis not seen in tumors from the other control groups (Fig. 6A). Panc-1 tumors exposed to RF fields after C225-AuNP treatment were significantly smaller after 6 weeks than tumors treated with C225-AuNP alone ($P = 0.0097$; Fig. 6B). In addition, cleaved caspase-3 was increased in Panc-1 tumors from mice treated with C225-AuNPs and exposed to the RF field compared with tumors from control mice (Fig. 6C and D). Tumor control was demonstrated after the first week

Fig. 2. C225 seems to quickly and uniformly bind to the surface of Panc-1 cells after treatment (durations listed at the top of each column), whereas C225-AuNP binds more slowly and in discrete, punctuate morphology consistent with an antibody conjugated to AuNPs. Blue: DAPI bound to DNA; Green: AF 488–labeled secondary antibody against human IgG.

Fig. 3. AF 647–labeled C225 demonstrates significant colocalization with the secondary anti-IgG, suggesting that the C225-AuNP construct remains intact after internalization into the cell (A). Although there is mild autofluorescence ($\sim 2\%$) in the far red channel (second column from left, confocal microscopy), using a fluorescent threshold of 7 arbitrary units (a.u.) shows that more than 78% of cells contain measurable levels of AF 647–labeled C225 regardless if AuNPs are conjugated. B, Based on fluorescence cell counting, the percentages represent the proportion of cells with fluorescence greater than 7 a.u. whereas the $P$ value compares the mean fluorescence intensity of each distribution to the negative control.
of treatment (treatment began between weeks 1 and 2, overall \(P = 0.004\); Fig. 6E).

The mice exposed to RF fields (with or without C225-AuNP treatment) did not show any evidence of behavior changes, gross injuries, or other signs of treatment toxicity during the experiment. Importantly, sections of the lungs, spleen, kidneys, and liver did not show any evidence of acute or chronic injury on extensive histopathologic examination despite the presence of AuNPs and RF field exposure (Fig. 6; Supplementary Fig. S2).

**Destruction of Capan-1 human pancreatic xenografts after PAM4-AuNP treatment and RF field exposure**

After demonstrating that C225-AuNP could effectively control pancreatic xenografts, we investigated this model further by utilizing a potentially more pancreatic cancer-specific antibody, PAM4, and a unconjugated AuNP + RF control arm (Fig. 6F). Capan-1 xenografts demonstrated statistically smaller tumors over the course of the experiment after PAM4-AuNP treatment, and RF field exposure began between weeks 1 and 2 (\(P = 0.035\); Fig. 6F). Interestingly, the unconjugated AuNP + RF control arm (Fig. 6F, blue line) demonstrated early effectiveness at decreasing tumor volumes, which was lost by week 5. However, PAM4-AuNP treatment without RF exposure did not significantly reduce the size of Capan-1 tumors from the untreated controls (Fig. 6). Similar to Panc-1 cancers, Capan-1 tumors treated with PAM4-conjugated AuNPs followed by RF field exposure were necrotic compared with control tumors treated

---

**Fig. 4.** Transmission electron microscopy shows internalization of AuNPs in Panc-1 cells after treatment with 100 \(\mu\)g/mL of AuNP-conjugated C225 for 3 hours (A). After RF field exposure, Panc-1 cells treated with C225-AuNP show significantly increased apoptosis (B, red bar). Statistical significance compares differences in the viable cell population (blue bar) between each group and the untreated control group on the basis of flow cytometry (B, far left group).

**Fig. 5.** Nude balb/c mice with pancreatic xenografts in the right flank were sedated and grounded to a copper plate with conducting copper tape in order to prevent excessive current inducing electrothermal injury (A). Tumors heat significantly more so than the mouse body due to C225-mediated AuNP delivery (B). In addition, 100 \(\mu\)g/mL of AuNPs in deionized water in an RF field whereas the conducting copper tape protects mice extremities (ears, tails, and paws) from excessive current and electrothermal injury.
with RF field exposure or PAM 4-targeted AuNPs alone (data not shown).

Again there was no evidence of injury to any of the selected organs (liver, spleen, kidneys, or lung) on histopathologic analysis in the experimental groups. Furthermore, there were no changes to gross behaviors or habits of the animals throughout the course of the experiment.

Discussion

The obvious goal, and major hurdle, of cancer therapy is to kill cancer cells without injury to normal or bystander cells, tissues, or organs. We have shown that RF fields can induce intracellular hyperthermic cytotoxicity with targeted AuNPs while controlling relatively large pancreatic cancer xenografts. Importantly, this occurred without any evidence of injury to selected normal tissues (liver, spleen, lung, and kidney), changes in animal behavior, or unexplained animal death. It is imperative that the AuNP and antibody remain conjugated until intracellular delivery. We are very confident that our constructs remain conjugated until delivery because of the optical absorption after sodium chloride challenge (Fig. 1), the diameters of the final constructs, and differences in immunofluorescence imaging.

RF field exposure in this situation was “whole-body” exposure. A consequence of this exposure is that the certain portions of animals within the field are at different electrical potentials. Variations in electrical potentials results in electrical currents that can produce a skin burn. Larger animals, however, have the body mass to absorb this “excess” current, whereas smaller animals (e.g., rodents) may have regions of increased electrical current with subsequent thermal injury to the ears, paws, and tails. To prevent this, we electrically grounded the animals’ extremities to the grounded plate of the RF device in a similar fashion to grounding pads used clinically for surgical electrocautery. Fortunately, we found this to be very effective without decreasing the ability to destroy the tumor, as the tumors were not directly isolated from the RF field. The bulk temperature of the tumors continued to rise (Supplementary Fig. S1) above the body surface temperature, consistent with bulk thermal transfer from intracellular AuNPs to cancer cells to surrounding tissues, which was visualized by the IR camera.

The in vitro experiments described herein were planned and carried out with the restrictions of the in vivo experiment in place. For example, raising the animal body temperatures above 41.5°C was presumed to be injurious to normal tissues. Therefore, the in vitro protocol was used with...
these limitations. Likewise, we investigated early apoptosis at a single time point. Previous studies have shown both apoptosis and necrosis at varying time points after RF field exposure depending on the construct involved and the RF field utilized (7, 8, 22). Finally, AuNPs of slightly different sizes were used to show the effect while keeping the final construct size similar.

Although C225 was predominately utilized in this series of experiments because of ease of conjugation, it may have increased amounts of adverse events due to high expression of EGFR on the skin and in bowel mucosa among other normal tissues (23, 24). C225-conjugated AuNPs were more effective than PAM4-conjugated AuNPs in destroying targeted tumors, but C225 as a targeting agent may have more adverse clinical events because of enhanced AuNP uptake in nonmalignant tissues. PAM4 seems to be much more pancreatic cancer specific and functions only as a pancreatic adenocarcinoma targeting antibody with no intrinsic cytotoxic or growth inhibition characteristic (18, 25, 26). Furthermore, even antibodies that fail as monotherapy for pancreatic cancer may function as an effective tool to deliver AuNPs. We did not include C225- or PAM4-only control groups in our animal studies because PAM4 does not affect cell proliferation and C225 alone has not been active against pancreatic cancer cells in the current in vitro studies or previous in vivo work (8, 25). Realistically, conjugating AuNPs to several different targeting molecules that attach to ligands differentially expressed in heterogeneous cancer cell populations will be a better approach; we are currently performing such studies in vitro and in vivo.

Enhancing thermal sensitivity of cancer cells may be achieved by incorporating a third component, such as a toxin or a cytotoxic drug, to the antibody-AuNP construct in order to potentiate the hyperthermic effect and impede cell repair mechanisms. In this case, the AuNP would act as scaffolding for 1 or more targeting proteins, therapeutic toxins, or chemotherapeutics to potentiate thermal toxicity, and it would also be the agent to produce heat from RF field exposure.

An obvious challenge in utilizing this therapy is the specificity of the targeting antibody as well as the non-specific internalization by the liver and the spleen. We demonstrated that neither the lungs nor the kidneys internalized significant amounts of AuNPs. We theorize that because the kidney and lungs act as “filtering” organs in addition to their primary functions, the small size of the AuNP constructs utilized herein permit passage of these nanoparticles without accumulation, although the development of liver toxicity will need to be closely monitored in the future. However, as we have demonstrated here, there was no evidence of liver injury despite clear presence of AuNPs and RF field exposure. We suggest that the regenerative characteristics of the normal hepatocytes tolerate hyperthermic treatment more so than other tissues, including tumors (27, 28). Furthermore, there is evidence that the majority of splenic and hepatic uptake of nanoparticles is by tissue macrophages without major induction of proinflammatory cytokines (29–31). Splenic macrophages or hepatic Kupffer cells that endocytose AuNPs may be affected by RF field treatments, but these cells are rapidly repopulated from circulating macrophage populations (32). Finally, there was no evidence of acute or chronic injury, as the bulk temperatures in the liver likely remained relatively normal.

Future experiments with this modality include the development of an orthotopic model as well as the investigations of micrometastatic disease. We have demonstrated that antibody-delivered AuNPs induce significant tumor destruction in a murine model of pancreatic carcinoma after RF field exposure.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Kristine Ash and Yolanda Brittain from the Department of Surgical Oncology, The University of Texas M. D. Anderson Cancer Center, for administrative assistance. They also thank the staff and animal care technologists of the Department of Veterinary Medicine and Surgery, specifically Maurice J. Dufilho, IV. In addition, we acknowledge Dr. Jared K. Burks, of the Flow Cytometry and Cellular Imaging Core, at the University of Texas M. D. Anderson Cancer Center (NCI Core grant CA16672), for assistance with confocal microscopy.

Grant Support

This work was funded from the NIH (U54CA143837), NIH M. D. Anderson Cancer Center Support grant CA016672, the V Foundation for Cancer Research, and an unrestricted research grant from the Kanzius Research Foundation (SAC, Erie, PA). E.S.G. is an NIH T32 research fellow (T32 CA09599).

Received 08/02/2010; revised 09/30/2010; accepted 10/01/2010; published online 12/07/2010.

References
