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LAB INVESTIGATION - HUMAN/ANIMAL TISSUE

Immunonanoshells for targeted photothermal ablation in medulloblastoma and glioma: an in vitro evaluation using human cell lines

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Abstract We are developing a novel approach to specifically target malignant brain tumor cells for photothermal ablation using antibody-tagged, near infrared-absorbing gold-silica nanoshells, referred to as immunonanoshells. Once localized to tumor cells, these nanoshells are extremely efficient at absorbing near-infrared light and can generate sufficient heat to kill cancer cells upon exposure to laser light. In this study, we evaluated the efficacy of immunonanoshells in vitro against both medulloblastoma and high-grade glioma cell lines. We used an antibody against HER2 to target gold-silica nanoshells to medulloblastoma cells, since HER2 is frequently overexpressed in medulloblastoma. We show that treatment with HER2-targeted nanoshells, but not non-targeted nanoshells, followed by exposure to laser light, can induce cell death in the HER2-overexpressing medulloblastoma cell line Daoy.2, as well as the parental Daoy cell line, which expresses HER2 at a moderate level, but not in dermal fibroblasts that do not express HER2. In an analogous set of experiments, we conjugated gold-silica nanoshells to an antibody against interleukin-13 receptor-alpha 2 (IL13Rα2), an antigen that is frequently overexpressed in gliomas. We demonstrate that these immunonanoshells are capable of inducing cell death in two high-grade glioma cell lines that express IL13Rα2, U373 and U87, but not in A431 epidermoid carcinoma cells that do not express significant levels of IL13Rα2. We believe that the use of antibody-tagged gold-silica nanoshells to selectively target cancer cells presents a promising new strategy for the treatment of central nervous system tumors that will minimize the damage and resulting toxicity to the surrounding normal brain.

Keywords Biophotonics · Glioma · HER2 · Interluekin-13 receptor · Medulloblastoma · Nanoshells · Nanotechnology · Near infrared

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Introduction

Primary brain tumors, including medulloblastoma and glioma, typically have a poor prognosis in both children and adults, particularly if the primary tumor cannot be completely resected. Currently available chemotherapy has done little to improve the outcomes for patients diagnosed with either a medulloblastoma or high-grade glioma. In addition, many patients who do achieve long-term survival frequently suffer significant morbidity related to current state-of-the-art therapeutic approaches. Thus, alternative strategies for the treatment of malignant brain tumors are needed. To this end, we have initiated development of an approach that uses antibody-targeted nanoshells for photothermal ablation of brain tumors.

Nanoshells consist of a spherical dielectric core nanoparticle surrounded by a thin metal shell [1]. By varying the size and composition of each layer of the nanoshell, it is possible to generate nanoshells that can either absorb or scatter light at a desired wavelength. We have used nanoshells with a silica core of approximately 100 nm and a 10 nm gold shell, which were optimized to have peak light absorption at 800 nm, which is in the near infrared range. This region of the electromagnetic spectrum is notable for

minimal absorption by water and biological chromophores [2]. Thus, light of this wavelength may penetrate deep into tissues with minimal disruption. In addition, systems have been developed that allow efficient treatment of the brain with near infrared (NIR) light [3–5]. The ability to treat the central nervous system (CNS) is an important consideration in the development of new therapies for medulloblastoma and glioma, as local recurrence is a frequent issue in these patients. In addition, since nanoshells are roughly one million times more efficient at converting NIR light into heat than conventional dyes such as indocyanine green [6], once localized to the tumor and exposed to NIR light, they can generate sufficient heat to induce cell death via thermal ablation [6].

Not only do gold-silica nanoshells have advantageous optical properties, but they have two other key features. First, their size allows them to accumulate within tumors due to the enhanced permeability and retention effect [7]. This mechanism, by which 60–400 nm particles accumulate in tumors, is thought to involve the leaky nature of the vasculature and reduced lymphatic clearance within tumors. Second, the chemical composition of nanoshells allows for the simple addition of targeting molecules, as well as coating the surface with polyethylene glycol (PEG), which can reduce both their immunogenicity and clearance. The utility of these features has been demonstrated by the eradication of subcutaneously grown tumors generated from colon cancer cells in mice treated with nanoshells and laser light [8].

In an effort to further enhance the specific localization of nanoshells to cancer cells, the nanoshells can be conjugated to antibodies or other targeting molecules. For example, we demonstrated previously that anti-HER2 tagged nanoshells bind selectively to HER2 overexpressing breast cancer cells in vitro [9]. Since HER2 overexpression occurs in a subset of medulloblastomas and correlates with metastasis [10] and poor prognosis [11–13], while the level of HER2 in the normal CNS is undetectable [14]; we examined the ability of HER2 targeted immunonanoshells to selectively target and induce photothermal ablation in medulloblastoma cells.

In contrast to medulloblastomas, high-grade gliomas less commonly express high levels of HER2. However, expression of interleukin-13 receptor alpha 2 (IL13Rα2) has been demonstrated in a high percentage of adult and pediatric high-grade gliomas, and this molecule is virtually absent from the normal brain tissue [15, 16]. The IL-13 receptor has shown promise as a target for novel anti-glioma therapies, including toxin-conjugated antibodies, targeted viruses, and tumor vaccines [17–19]. Therefore, we evaluated the ability of an antibody to IL13Rα2 to target nanoshells to high-grade glioma cells and selectively induce photothermal ablation.

Materials and methods

Cell lines and cell culture

All of the cell lines used were of human origin. The medulloblastoma cell lines, Daoy (ATCC, Manassas, VA) and Daoy.2, a clonal derivative of Daoy that overexpresses HER2 [10] (a gift from John Kim, Texas Children's Hospital, Houston, TX) were maintained in RPMI 1640 medium (Gibco—Invitrogen Corp., Carlsbad, CA). The glioma cell lines U373 (ATCC) and U87 (ATCC) were grown in RPMI 1640 and Dulbecco's Modified Eagle's Medium (DMEM) (Gibco—Invitrogen Corp.), respectively. The epidermoid carcinoma cell line A431 (ATCC) was grown in DMEM. Human dermal fibroblasts (HDF) (ATCC) were grown in DMEM. All media were supplemented with 10% fetal bovine serum, and the cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

Gold-silica nanoshell fabrication

Nanoshells with peak optical absorption efficiency in the NIR range (800 nm) were fabricated as described previously [20]. Briefly, silica cores with a diameter of approximately 100 nm were generated using the Stöber method [21]. Terminal amine groups were added to the surface using aminopropyl triethoxysilane. Gold colloid, made as described by Duff et al. [22], was then electrostatically adsorbed to the surface. Lastly, additional gold was reduced onto these nucleation sites using $HAuCl_4$ and potassium carbonate.

Conjugation of antibodies and polyethylene glycol (PEG) to nanoshells

Antibodies to HER2 (Ab-4, clone N12, NeoMarkers, Fremont, CA), IL13Rα2 (clone B-D13, Cell Sciences, Inc., Canton, MA) or control antibody (Ab-1, clone NCG01, NeoMarkers) were mixed with orthopyridyl-disulfide-poly(ethylene glycol)-N-hydroxysuccinimide ester (OPSS-PEG-NHS, 2000 MW, Nektar, San Carlos, CA) and allowed to bind overnight at 4°C. The PEG-conjugated antibodies were then added to the nanoshells for 1 h prior to addition of PEG-thiol and the reaction was allowed to proceed overnight at 4°C. The immunonanoshells were then centrifuged in 1 ml aliquots at 500 g for 5 min to remove unbound antibody and PEG. Absorption spectra were obtained using a Cary 50 BIO UV/Vis spectrophotometer (Varian, Inc., Palo Alto, CA), and the nanoshells were diluted in cell culture media to a final peak absorbance of 5 immediately prior to use.

Transmission electron microscopy of immunonanoshells

Immunonanoshells, prepared as described above, were incubated with gold-labeled anti-mouse IgG (Molecular Probes - Invitrogen Corp.) for 1 h. Non-specific reaction sites were blocked with a 3% solution of bovine serum albumin. Unbound IgG was removed by centrifugation. Samples were mounted on copper grids and imaged with a JEOL 2010 transmission electron microscope (JEOL Ltd., Tokyo, Japan).

In vitro photothermal nanoshell therapy

Cells were seeded in 24 well plates and grown until nearly confluent. Cells were then washed once with phosphate buffered saline (PBS) and 7.2×10^9 nanoshells in 0.5 ml of cell culture media were added to each well. After a one-hour incubation, the cells were washed three times with PBS to remove the unbound nanoshells. Then an approximately 2 mm diameter spot in each well was exposed to a laser (Coherent Inc., Santa Clara, CA) at 800 nm and 80 W/cm^2 for 2 min. The next day, the cells were stained using the Live/Dead viability/cytotoxicity kit (Invitrogen Corp., Carlsbad, CA) and were visualized using a Ziess Axiovert 135 fluorescence microscope (Ziess, Thornwood, NY). A schematic of this experimental design is shown in Fig. 1. Each treatment group was performed in duplicate or triplicate, and all experiments were repeated at least twice. The photomicrographs that are shown are representative of the results from these multiple experiments.

Results

Production of immunonanoshells

Gold-silica nanoshells were fabricated as described as above, and aliquots were then conjugated to either PEG alone, in order to minimize non-specific binding of the nanoshells, or the combination of a targeting antibody and PEG. Figure 2 shows that the absorption spectra of the bare nanoshells, PEGylated nanoshells, and antibody-conjugated nanoshells were nearly identical. The PEG and antibody alone did not have detectable absorption in the NIR range. Thus, the optimized optical properties of the nanoshells were not altered by the addition of PEG or the targeting antibodies.

A transmission electron microscope image of an antibody-conjugated nanoshell is shown in Fig. 3. For this image, a secondary antibody conjugated with gold beads was bound to the anti-HER2 antibodies for visualization. In addition, the coating of PEG can be seen.

HER2 targeted nanoshells in medulloblastoma

As shown in Fig. 4, bare nanoshells, which can adsorb to cell surfaces non-specifically, induced cell death in the area treated with the laser in both the medulloblastoma cell line,

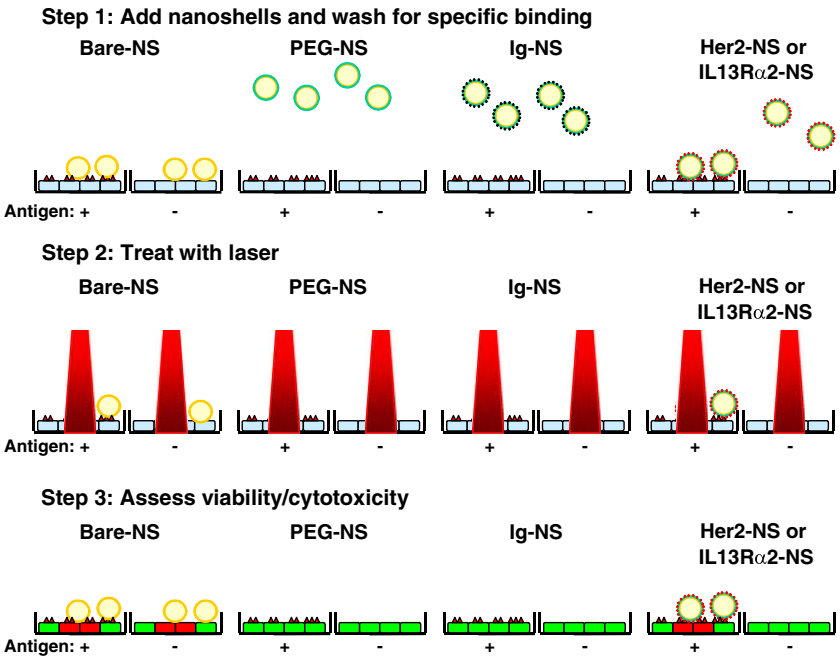

Fig. 1 Schematic diagram of the experimental design used to test the specificity of photothermal ablation achieved with antibody targeted nanoshells in vitro

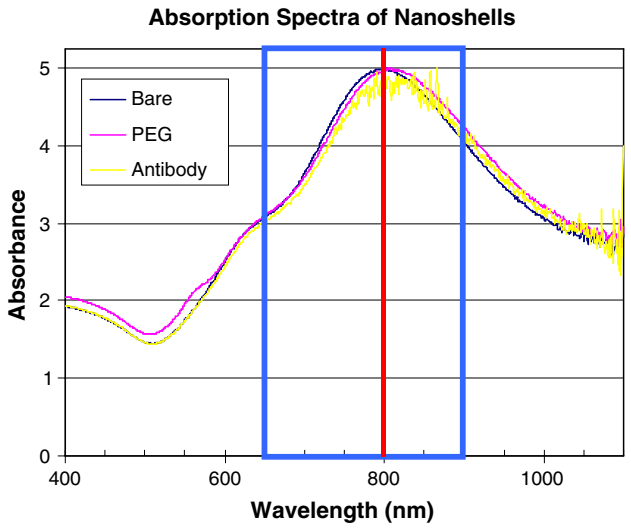

Fig. 2 The absorption spectra for bare nanoshells, PEGylated nanoshells, and antibody conjugated nanoshells was obtained using a spectrophotometer. The data is normalized to a peak absorbance of 5, to which all nanoshells were diluted prior to use. The red line at 800 nm indicates the wavelength of the laser that was used for our treatments. The blue box indicates the near infrared window

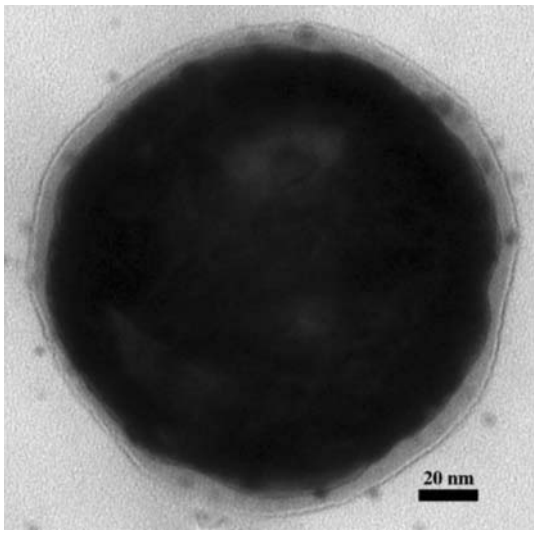

Fig. 3 A transmission electron microscope image of a gold-silica nanoshell that has been PEGylated and conjugated to anti-HER2 antibodies. The antibodies have been tagged with gold beads for visualization.

Daoy, and in the HDF control cells. However, the nanoshells tagged with anti-HER2 antibody induced cell death in the Daoy cells, which express low but detectable levels of HER2, but not in the HDF cells, which do not express detectable HER2. The specificity of binding was confirmed by the observation that neither PEGylated nanoshells nor nanoshells tagged with a control antibody induced cell death in the Daoy cells. Phase contrast images demonstrate nanoshell binding correlates with cytotoxicity following laser treatment. Similar results were seen with the HER2 overexpressing cell line Daoy.2 as with the Daoy cells (data not shown).

IL13Rα2 targeted nanoshells in glioma

Analogous to the experiments using medulloblastoma cells, we evaluated whether anti-IL13Rα2-tagged nanoshells could target glioma cells that express IL13Rα2. As seen in Fig. 5, bare nanoshells induced cell death in the area treated with the laser in both the IL-13Rα2 expressing glioma cell line, U373, and in A431 cells, which do not express detectable IL-13Rα2 [16]. However, the nanoshells tagged with an antibody specific for IL-13Rα2 induced cell death in the U373 cells but not in A431 cells. The specificity of binding was confirmed by the observation that PEGylated nanoshells did not induce cell death in either cell type. Similar results were seen with the U87 glioma cell line, which expresses high levels of IL-13Rα2 [16], as with the U373 cells (Fig. 5c).

Discussion

Improved therapies for brain tumors including medulloblastoma and glioma are clearly required if we are to achieve the expectations of long term survival and high quality of life including normal cognitive function for patients with these diagnoses. In an effort to develop a therapeutic strategy that selectively targets the tumor yet is potent enough to induce cell death in the cancer cells, we have examined anti-HER2 and anti-IL13Rα2 antibody tagged gold-silica nanoshells as a potential tool for thermal ablation of cancer cells using a NIR-laser. Our in vitro experiments demonstrate that this strategy can selectively kill medulloblastoma cells that express HER2 without killing cells that do not express HER2. Not only was this effect observed in Daoy.2 cells, which were transfected to express high levels of HER2, but also in the Daoy parental cell line, in which the expression of HER2 is much lower. Likewise, we demonstrated using IL13Rα2 targeted nanoshells we could selectively kill high-grade glioma cells that express IL13Rα2 without killing cells that do not express IL13Raα2.

Although the standard approach to the treatment of most CNS tumors is multi-modal including surgery, radiation therapy and chemotherapy, brain tumors remain a tremendous treatment challenge [23]. Many primary brain tumors are not amenable to a gross total resection, as even under the best of circumstances tumor tissue is intimately involved with vital structures within the brain. Although radiation therapy is beneficial in the treatment of many

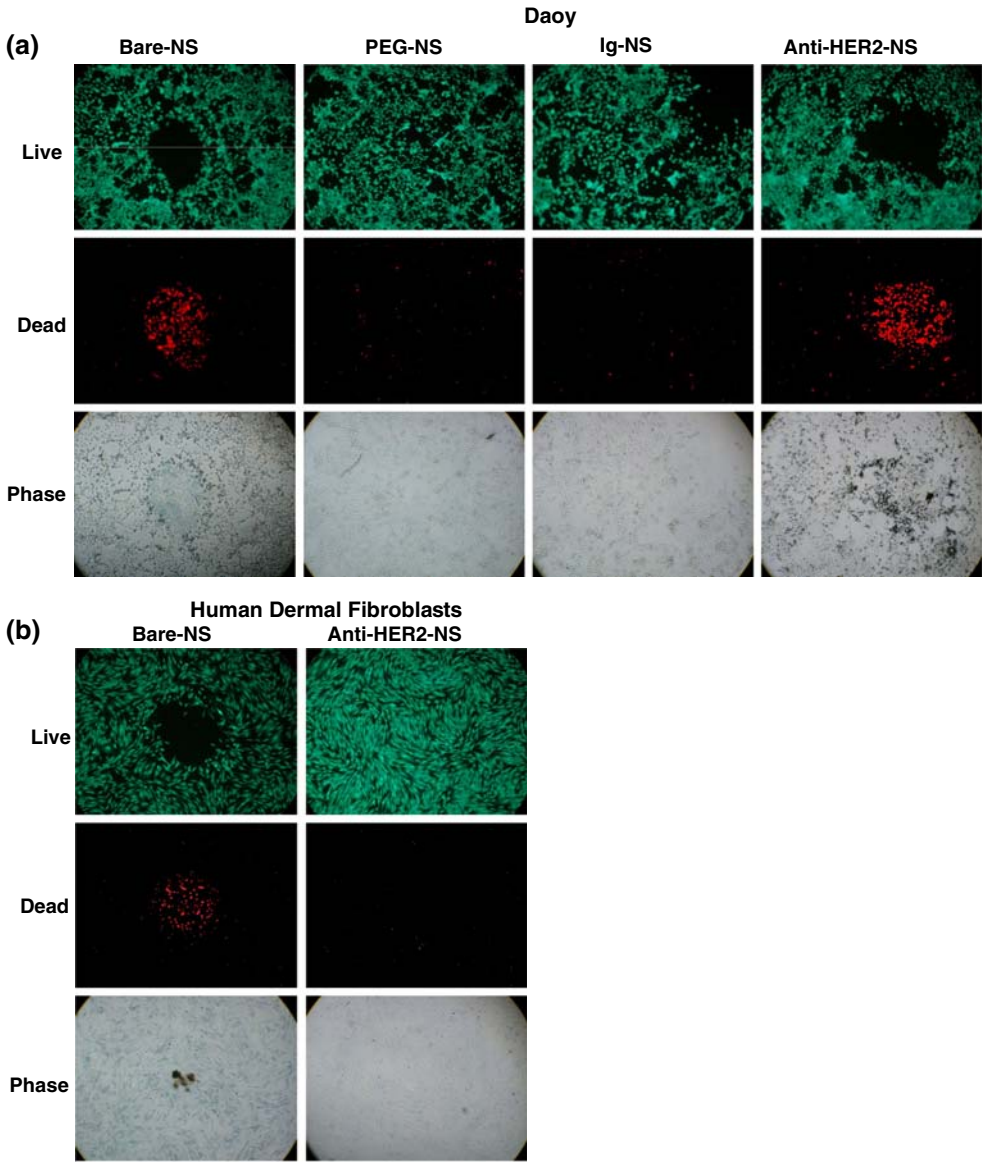

Fig. 4 HER2 positive Daoy medulloblastoma cells (**a**) and HER2 negative HDF (**b**) were treated with nanoshells without PEG or antibody (Bare-NS), with PEGylated nanoshells (PEG-NS), or with PEGylated nanoshells conjugated to a control antibody (Ig-NS) or anti-HER2 antibody (anti-HER2-NS). The cells were then washed to remove unbound nanoshells and a 2 mm diameter area was treated with a NIR laser for 2 min. The following day, cells were stained with calcein AM and ethidium homodimer and imaged using a fluorescent microscope. Thus, in the treatment groups where the nanoshells remained bound and induced cell death, a circular area where the laser was applied does not take up the green calcein AM (Live—top row), but does stain red using the ethidium homodimer (Dead—middle row). Binding of the nanoshells can be visualized in the phase contrast images (bottom row)

malignant central nervous system tumors, the benefit is generally of limited duration for patients with high-grade glial tumors [24]. In addition, radiation therapy is associated with the potential for devastating neurocognitive and neuropsychological seqeulae, particularly in the pediatric population, and the development of second malignant neoplasms [25]. Chemotherapy, although of benefit in many children with medulloblastoma and a subset of patients with gliomas, has many short and long term side effects [26, 27]. Thus, new, effective and less toxic treatment approaches are needed.

Our therapeutic approach using targeted immunonanoshells for thermal ablation has several potential key advantages over standard treatment modalities, as well as other strategies that are in development, such as targeting peptides conjugated to toxins. First, while cellular resistance to chemotherapy, and potentially other toxins, can be challenging to overcome, resistance to thermal ablation

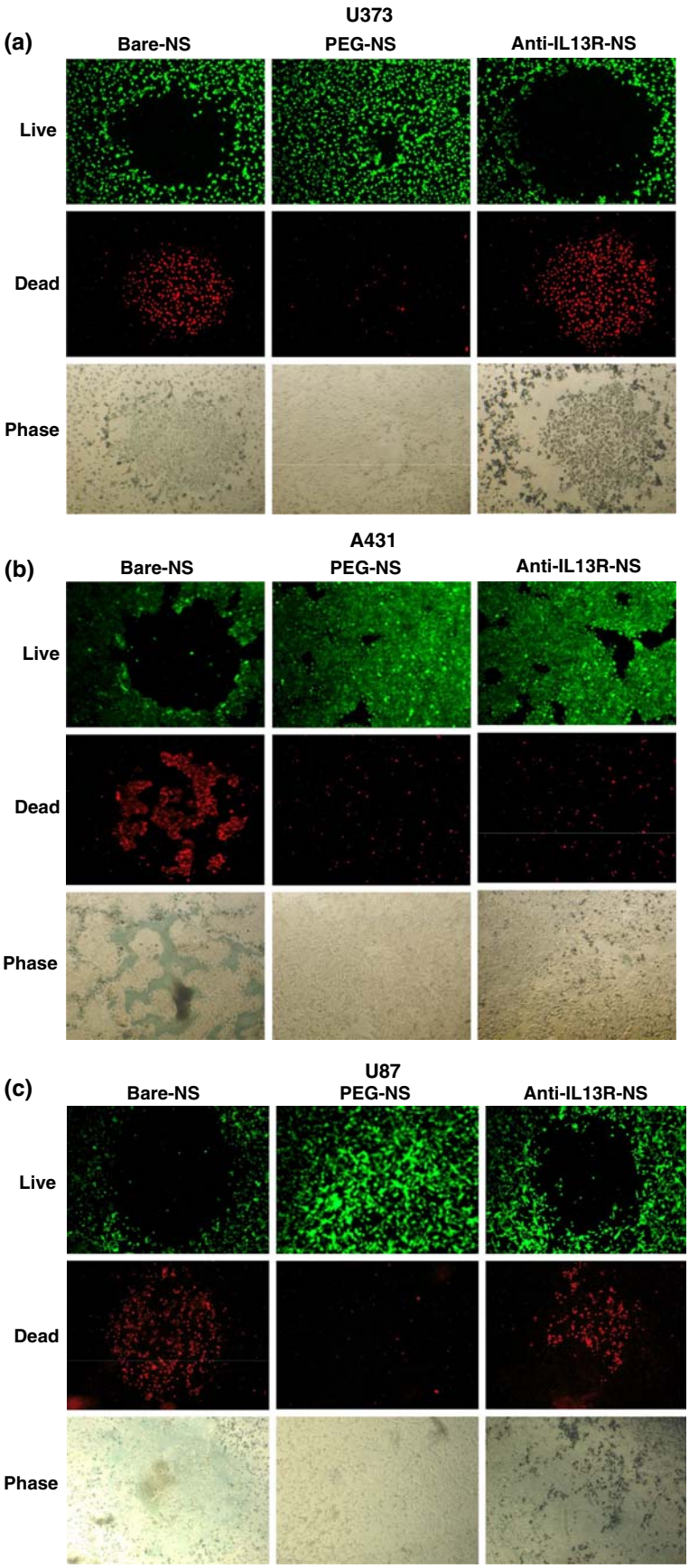

Fig. 5 IL13Rα2 positive U373 glioma cells (**a**) and IL13Rα2 negative A431 cells (**b**) were treated with nanoshells without PEG or antibody (Bare-NS), with PEGylated nanoshells (PEG-NS), or with PEGylated nanoshells conjugated to anti-IL13Rα2 antibody (anti-IL13R-NS). The cells were then washed to remove unbound nanoshells and a 2 mm diameter area was treated with a NIR laser for 2 min. The following day, cells were stained with calcein AM and ethidium homodimer and imaged using a fluorescent microscope. Thus, in the treatment groups where the nanoshells remained bound and induced cell death, a circular area where the laser was applied does not take up the green calcein AM (Live—top row), but does stain red using the ethidium homodimer (Dead—middle row). Binding of the nanoshells can be visualized in the phase contrast images (bottom row). In panel (**c**), the results are shown for the U87 glioma cells, which also express IL13Rα2

would be unlikely. In addition, while cancer cells may change their expression of surface markers, the chemistry of the nanoshells allows for the simple addition of nearly any targeting molecule. Further, we have used a number of targeting molecules to date, and they have not been found to significantly alter the absorption peak of the nanoshells. In contrast, the function of targeting peptides conjugated to toxins has been shown to be affected by the linkage of the two components [28]. In addition, particles in the size range of the nanoshells have been shown to accumulate in tumors even without specific targeting molecules due to the enhanced permeability and retention effect [7]. The immunonanoshells also do not require entry into the cell in order to induce cell death. Also, the composition of the nanoshells is of substances (silica, gold, PEG, and targeting antibodies) that have been used previously as pharmacological agents. Furthermore, as was demonstrated in our in vitro experiments, the toxicity of the nanoshells is limited to areas that are also exposed to NIR light. The dual requirement for the presence of both nanoshells and laser light to induce cellular death is particularly promising for use in brain tumors, for which local recurrence is often the most serious risk and the surrounding normal tissues perform critical functions.

In conclusion, we have demonstrated that immunotargeted nanoshells can selectively induce cancer cell death in vitro. In vivo studies are currently underway in orthotopic brain tumor models in mice using intravenous injections of immunonanoshells, and the first human clinical trials using gold-silica nanoshells in other non-CNS tumors will commence in the immediate future. The results of these studies will be crucial for defining the potential benefit of this therapeutic strategy for patients with central nervous system tumors.

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