A strategy for blood biomarker amplification and localization using ultrasound

Aloma L. D’Souza, Jeffrey R. Tseng, Kim Butts Pauly, Samira Guccione, Jarrett Rosenberg, Sanjiv S. Gambhir, and Gary M. Glazer

Departments of Radiology and Bioengineering and Molecular Imaging Program, Stanford University, Stanford, CA 94305

Blood biomarkers have significant potential applications in early detection and management of various diseases, including cancer. Most biomarkers are present in low concentrations in blood and are difficult to discriminate from noise. Furthermore, blood measurements of a biomarker do not provide information about the location(s) where it is produced. We hypothesize a previously undescribed strategy to increase the concentration of biomarkers in blood as well as localize the source of biomarker signal using ultrasound energy directly applied to tumor cells. We test and validate our hypothesis in cell culture experiments and mouse tumor xenograft models using the human colon cancer cell line LS174T, while measuring the biomarker carcinoembryonic antigen (CEA) before and after the use of ultrasound to liberate the biomarker from the tumor cells. The results demonstrate that the application of low-frequency ultrasound to tumor cells causes a significant release of tumor biomarker, which can be measured in the blood. Furthermore, we establish that this release is specific to the direct application of the ultrasound to the tumor, enabling a method for localization of biomarker production. This work shows that it is possible to use ultrasound to amplify and localize the source of CEA levels in blood of tumor-bearing mice and will allow for a previously undescribed way to determine the presence and localization of disease more accurately using a relatively simple and noninvasive strategy.

Blood biomarkers have the potential to have a significant impact on clinical medicine because they can potentially predict risk for disease, signal the initial presence of disease and its recurrence, help in the selection of patients for a particular treatment, and monitor response during and after treatment. Consequently, there has been a large body of work attempting to discover and validate serum biomarkers, particularly for the detection of cancers (1–10). Despite the numerous investigations exploring the clinical use of biomarkers and a large increase in the number of potential biomarkers identified, few recently identified blood biomarkers are used for clinical care, and their rate of introduction into the clinical environment is actually falling (8, 11). Additionally, the reproducibility of studies using blood biomarkers for the detection of cancer has been poor (4, 12).

Two fundamental problems hinder current serum biomarker research: the concentration of biomarker signal in the blood is often very low, and the localization of the site(s) of origin of the biomarker signal cannot be determined solely from measurement of blood levels of the biomarker. The most desirable biomarkers are produced within a specific tissue but are present in extremely low concentrations in the plasma. The concentrations in plasma are often so low that biomarker measurements can either be difficult or impossible to differentiate from the noise of the measurement process and system. This markedly hinders the efficacy and reliability of biomarker measurement in blood. Furthermore, even when a disease-specific (e.g., tumor) biomarker can be reliably identified by its presence in the blood, there is no information about its specific anatomical site of origin or spatial localization. So, in the hypothetical case of a recently identified serum biomarker that reliably detects a cancer, the patient and his or her physician might be dealing with information about the presence of a tumor without knowing precisely where it is located in the body.

We hypothesized that both of these fundamental issues could be addressed by applying ultrasonic energy to potential tumor sites. Ultrasound with frequencies from 20 kHz to 16 MHz has been reversibly shown to compromise the integrity of cell membranes, causing an increase in permeability, which has been widely used as a method of intracellular delivery of macromolecules (13–18). The precise mechanism of this action is not clear, but it has been shown to cause the formation of transient pores attributable to membrane disruption (15, 19, 20). Because multiple copies of biomarker proteins are present either on the surface of tumor cells, within cell membranes, or within the cytoplasm of these cells, it is possible that these copies can be released from tumor cells by the appropriate application of ultrasonic energy to the tumor, potentially providing biomarker amplification in the extracellular space fluid or in blood. Our hypothesis is also based on the fact that ultrasonic energy can be deposited in a spatially localized way, potentially releasing biomarkers only when the energy is applied to a tumor or portions of a tumor. We chose the initial biomarker used for routine clinical cancer care in the detection and recurrence of colon cancer, carcinoembryonic acid (CEA) (2, 21), to test our hypothesis at a low 1-MHz frequency of ultrasonic energy. Our results in cell culture and in tumor-bearing living mice form the basis of this proof-of-concept report.

Results

Cell Culture Studies. Effects of cell death on CEA release. LS174T cells were exposed to varying ultrasound intensities to study the correlation between CEA levels released in the media and the amount of cell death. CEA levels progressively increased with the percentage of cell death, with a correlation of 0.97 (Fig. 1A). To ensure that the CEA released from cells was not entirely attributable to cell death in our studies, we determined the ultrasound conditions for which we obtained less than 5% total cell death. Cells were exposed to 1-MHz ultrasound frequency at various power levels (0.1, 0.3, 0.5, 0.8, 1.0, and 1.5 W/cm²) and at different duty cycles (10, 20, 50, and 100%) for 30 sec. Power is defined as the energy (W) emitted divided by the area (cm²) of the emitting surface, and duty cycle is defined as the percentage of time the sound wave is delivered. The amount of cell death determined showed a relative increase with increase in power and duty cycle (Fig. 1B). At power levels below 1 W/cm² and duty cycle below 20%, the cell death in cell culture was...
estimated to be less than 1%. This result shows that a large amount of the detected CEA in media at high ultrasound power deposition is likely attributable to cell death. To test whether CEA release could be achieved at a low level of cell death, we subsequently used ultrasound at a low power level (0.3 W/cm²) and at a short duty cycle (10%).

**Time release of CEA from cells in culture treated with ultrasound.** To study the release of CEA, cells were exposed to a constant ultrasonic pulse (1 MHz, 0.3 W/cm², 10% duty cycle) for 30 min (n = 5). A steady accumulation of CEA in the media with time was observed (Fig. 2). Within 30 min, there was a 3- to 4-fold increase in the CEA levels when compared with untreated control samples (n = 2) for the same time period. This increase in accumulation was significant in comparison with the controls at 20 and 30 min (P < 0.036 for both). Cell death averaged across the samples was 0.5% for the controls and 2.2% for the treatment group at the 30-min time point. The results of this experiment were repeated and confirmed.

**Living Mice Studies.** To prove our hypothesis in living subjects, we used xenograft s.c. tumors of LS174T cells in nude mice (n = 7 per treatment group, n = 28 total). The clearance of CEA from blood in nude mice has been previously well studied. Human CEA extracted from tumors shows a clearance in nude mice of about 90% within 10 min, 95% by 30 min, and 98% by 2 hours, whereas CEA extracted from human serum has a clearance of 2.5 hours when injected in nude mice (22). In accord with these results, which we confirmed, all treatments and blood collections were done within 10–15 min after the initial energy deposition.

Four ultrasound treatments were undertaken at 1 MHz for 6 min: no power or 0 W/cm², 4 W/cm² at 20% duty cycle, 6 W/cm² at 20% duty cycle, and 2 W/cm² at 50% duty cycle. Before implantation of the cells, the mice were treated with ultrasound as their own internal “no-tumor” controls (Fig. 3, control 1). As a further control, the ultrasound treatment was done on the tumor-bearing mice on a non–tumor-bearing site (flank opposite tumor implantation) after implantation and growth of the tumors (Fig. 3, control 2). Both of these controls showed no significant change in the levels of CEA in blood with the ultrasound treatments (P > 0.689 and P = 0.538, respectively), and there was no significant difference between the 2 controls (Fig. 4; P < 0.183). For the treatment group, ultrasound was applied directly to the site of the tumor with the ultrasound transducer positioned over the s.c. tumor (Fig. 3). The mean tumor volume was 0.499 ± 0.01 cm³ (SEM). The 21.5-mm transducer used has a well-collimated beam of energy, which penetrates tissue to a depth of 10–15 mm and measures about 11 mm across (Sonitron 2000; Rich-Mar Corporation), allowing most of the tumor to be exposed to the energy.

At 20% duty cycle, which would not be expected to cause much cell death or optimal CEA release on the basis of cell culture experiments, the use of power of 6 and 4 W/cm² did not show significant increase in CEA release when compared with controls (P < 0.81 and P < 0.99, respectively) (Fig. 4). To see if an increase in the duty cycle would cause a substantial release of CEA, 2 W/cm² at 50% duty cycle for 6 min was used and showed a significant increase in CEA levels after ultrasound treatment when compared with controls 1 and 2 (P < 0.03 for both) (Fig. 4). Preliminary experiments done with higher power levels at 50% duty cycle caused superficial burns on the skin and were not pursued.

Cell death attributable to ultrasound treatment in the s.c. tumors was not easily studied. Areas of necrosis were present in most tumor sections, and ultrasound-induced necrosis was not clearly differentiable from tumor necrosis. Sections stained for apoptosis, using caspase-3 staining, showed no marked differences in the area positively stained within the total area when comparing the 0 W/cm² (0.89% of total area) with tumors that had a positive release of CEA at 2 W/cm² (0.51% of total area) (n = 5 fields per tumor section, n = 7 tumors per treatment group). Overall, the caspase-3–stained cells were less than 2% of the area of the tumor sections (4 W/cm² = 0.83% and 6 W/cm² = 1.78% of total area).
increase in power (P at 20% duty cycle did not show a significant increase in the CEA levels with Fig. 4. Release of CEA from tumors treated with ultrasound. The s.c. LS174T tumors (n = 7 per group) exposed to 6 min of ultrasound at 0, 4, and 6 W/cm² at 20% duty cycle did not show a significant increase in the CEA levels with increase in power (P < 0.81 and P < 0.99, respectively). At 2 W/cm² at 50% duty cycle for 6 min, there was a significant increase in CEA release when compared with control 1 or control 2 (*P < 0.031 for both). Controls 1 and 2 did not show any significant increase in CEA release (P < 0.689 and P < 0.538, respectively). Error bars shown are SEM.

Fig. 3. Schematic setup of ultrasound treatment in living mice. Studies in living mice were done in 3 groups. Control 1: ultrasound was applied to mice with no tumors. Control 2: ultrasound was applied to a non–tumor-bearing region of mice with tumors. Treatment: Ultrasound was applied directly to the site of the tumor. Posttreatment minus pretreatment levels of CEA were hypothesized to increase in the treatment group because of the release of CEA into the bloodstream from the tumor.

Discussion

Both the cell culture and living mice results presented here support our hypothesis that ultrasonic energy applied to tumors can release protein biomarkers from the tumor, amplifying biomarker signals measured outside of the cells. In the live mouse, enough biomarker was released into the bloodstream to provide signal amplification so that blood levels of the biomarker were significantly elevated beyond baseline measurements. Also, our work in mice has proved the hypothesis that additional signal from the tumor-specific biomarker CEA was released only when ultrasonic energy was deposited in the tumor and was not released when the energy was deposited in normal tissue remote from the tumor. Thus, ultrasonic energy appropriately targeted and deposited in macroscopic tumors can potentially obviate major current limitations of serum biomarkers by providing a means for both amplification of the biomarker signal and localization of the site of biomarker residence.

There are a number of clinical applications that could be pursued given the results of our study. One very common problem in clinical practice is the detection of a lesion that is incidentally found on computed tomography (CT) or MRI studies performed for another purpose (23). In patients with existing malignancies, these “incidentalomas” commonly require further study, biopsy, or even surgical removal. The technique we describe could potentially be used in patients whose malignancies harbor biomarkers to distinguish between metastatic disease (which would release biomarker) and benign incidental lesions such as adenomas. Another clinical application that could be readily pursued is the characterization of a dominant lesion (e.g., in the breast) as benign or malignant. Our findings also have the potential to be used for proof of disease recurrence in patients who have tumors that produce biomarkers and who have a soft tissue mass on CT or MRI studies. In fact, patients like these could potentially have a low-intensity image-guided ultrasound study to prove disease recurrence, followed by a high-intensity focused ultrasound treatment to ablate the lesion.

Although there are many potential clinical applications enabled by our research, there are 4 main limitations to our study that deserve further comment. These limitations involve the use of a single biomarker, the use of s.c. macroscopic tumors, the use of a species-specific model, and the application and optimization of ultrasound energy deposition. It is important to point out that we used a single biomarker (the protein CEA) for these proof-of-principle studies, and there is no assurance that other protein biomarkers will yield similar results. For example, the plasma half-life in nude mice of human serum-extracted CEA is 2.5 hours (22), which is favorable for our study because the CEA released by our intervention was measured and detected before it was markedly degraded by proteases and cleared from the circulation. Other proteins with short plasma half-lives may present problems for this method. Another limitation in the use of CEA from LS174T cells is that it is one of the colon cancer cell lines with the highest CEA content compared with other cell lines (599.4 ng per 10⁶ cells) (24); some other tumor cells and biomarkers may have far less content per cell, thus requiring more total energy deposition. Also, we did not extensively study the biophysics of the ultrasonic intervention; thus, we are not certain whether most of the amplified signal came from the cell surface or from the cytoplasm of the cells that received energy deposition. This is an issue that could become important for biomarkers that are disproportionately located at a single cellular site.

In the clinical environment, there is emerging consensus that single plasma biomarkers will not prove effective for the detec-
tion or management of disease and that multiple panels of biomarkers will be required (2, 7–9). This consensus seems to be based on the fundamental biomarker problems our study addresses, and we are uncertain about how this issue will unfold in the future. Despite differences related to plasma half-lives and copy numbers, we believe it will be possible to amplify and localize signals from multiple biomarkers that reside within, or on, the surface of cells.

Differences in the accessibility of biomarkers to blood once they are released also affect the results of insonification. For a biomarker to be measured in the blood, it needs to be released from the tissue of origin and find access to the bloodstream. Results of biomarker release from the s.c. tumor experimental model we used may not be directly generalizable to the more common situations of tumors that develop within the soft tissues of organs in living systems. It is known that the microvasculature environment between the 2 situations can be different, and this may cause differential access of released biomarkers to the bloodstream. For example, orthotropic tumor models are known to have a higher macromolecular vascular permeability than s.c. tumors, which would allow for greater access of the biomarker to the blood (25, 26). Future studies will need to look at orthotropic tumor models and the release of biomarkers into the blood to examine this issue more carefully. Also, although the work in living mice reached clear statistical significance when appropriate ultrasound energy was used, it was more variable than the cell culture experiments. One major source of variability was that the number of viable tumor cells after tumor implantation and development was likely significantly different in each of the mice; thus, the amount of biomarker available for amplification differed among animals.

Another limitation of our study is that we insonicated macromolecular tumors, and we have no evidence that the method could be applied to tumors that are smaller and earlier in their state of evolution. Microscopic tumors that cannot be imaged in vivo would be particularly problematic; thus, they might not be amenable to our technique. It is possible, but highly speculative, that our method could be applied to an area that appears morphologically normal by imaging but that releases biomarkers, permitting the detection of disease that has not yet become macroscopic. For example, a possible scenario for its application includes a patient with high risk for cancer having a baseline blood draw for biomarker measurements and then having a repeat blood draw after deposition of ultrasound energy in an area of possible tumor (e.g., breast). In this way, the patient’s own pre-ultrasound biomarker level is compared with the post-ultrasound level, allowing potentially higher discriminatory capacity and spatial localization of possible tumor. If no tumor were present at the site of interest, no significant blood biomarker increase would be detected. This study would build on our mathematical modeling of early cancer detection through blood biomarker levels (10) because it allows each patient to be his or her own control before and after ultrasound treatment.

The mouse is a useful model to prove the feasibility of our approach; whether the results can be generalized to other species, including humans, is always uncertain. We are confident that the general concept of biomarker amplification after insonication will remain valid. However, the measurement of biomarker release will likely be highly affected by differences among species regarding proteases, which degrade biomarkers at varying rates in the blood, as well as by differences in blood volume among species. We have recently started to model some of these issues mathematically to relate blood biomarker levels to tumor volume (10). For example, the blood volume of the mouse is small compared with that in humans. After insonication, biomarkers released from cells would be far more diluted in humans than in mice; thus, changes in biomarker concentration might be undetectable after insonication. However, focused ultrasound systems, which differ and are improved compared with the ultrasound system we used, have been optimized for humans recently (27–30) and should help in signal amplification (and localization). Also, any improvements in the signal detection of the biomarker by techniques such as mass spectroscopy and nanosensors should prove helpful in obviating this limitation (7).

Furthermore, our research on the use of ultrasound to perturb tumors and amplify biomarker signals is dependent on the ultrasonic energy reaching the tumor. This requires an “acoustic window” from the transducer to the tumor. Ultrasound is reflected at air-tissue interfaces because of differences in acoustic impedances. In addition, ultrasound is highly attenuated by bone. Therefore, regions of living systems that lack acoustic windows include the lung, or any lesion completely surrounded by air, as well as regions surrounded by thick cortical bone. Fortunately, acoustic windows can generally be found for nearly all regions except the brain, spine, lung, bowel, and within bone; thus, the method we propose has relatively few limitations because of the absence of acoustic windows for insonication.

Also, we did not study how much of the released CEA came from live cells as opposed to cells that may have been killed because of the application of ultrasonic energy. The cell culture work clearly showed that cells killed by the deposition of ultrasound energy released very large amounts of CEA. This is important because there would be little objection to killing tumor cells with ultrasound in the clinical environment, and in this scenario, we can expect large signal amplification that might permit signal detection in blood. For our work in living mice, we elected to deposit relatively smaller amounts of energy to minimize cell death yet achieve signal amplification. This balancing of cell death and signal amplification will need to be worked out for clinical application of our technique.

A major source of variability in vivo was attributable to the specifics of the ultrasound application. Ultrasonic energy was manually applied by placing the transducer over the tumor; however, the ultrasonic coupling to the surface was less than optimal because of the application of a rigid flat transducer surface to a curved area of the tumor and of the animal. This introduced considerable variability into the results. The focal zone of the ultrasound energy deposition was not shapeable, but it was delivered uniformly through the tissue to a depth of a few centimeters; this could be considered to be suboptimal compared with what can be achieved with better ultrasound instrumentation.

Although we did not perform an extensive optimization of the energy deposition specifics in terms of the length of energy application or the intensity of the energy used and its correlation with biomarker amplification, many of these limitations can be overcome with the use of clinically available equipment that has been called “focused ultrasound” using image-guided magnetic resonance (MR) thermometry (27–29). This equipment is available in many academic departments of radiology throughout the world. Using this equipment, precise amounts of ultrasonic energy can be deposited at specific locations within an MRI-visible tumor, and MR thermometry can measure in nearly real time the temperature of the tissues insonicated. In this way, specific locations of tissue can be altered in a graded way using ultrasonic energy with feedback during the experiment from image-localized temperature readouts. We are beginning to pursue studies using focused ultrasound in a mouse system to control experimental variables better and to optimize biomarker release.

We envision a fairly clear pathway to explore clinical application of ultrasound for biomarker localization and release. Image-guided focused ultrasound has been used for the past decade to ablate tumors in humans; thus, many of the potential pitfalls of using ultrasound to deposit energy locally in patients have already been overcome. In fact, the United States Food and Drug Administration granted approval of MR-guided focused...
ultrasound to treat uterine fibroids in 2004 (31). Since that time, thousands of patients have been treated using this technology for tumors, including those of the uterus, breast, and brain and metastatic disease of the bone (27, 32, 33). These treatments, which use high-energy ultrasound for tumor ablation, have proved quite safe, with adverse events being quite uncommon (31). We do not anticipate significant safety concerns for the application of ultrasound to release biomarkers, because, according to our study, considerably lower energy will be used than in applications to ablate tumors. Furthermore, because MR-guided focused ultrasound systems are already commercially available, there should be little delay in adapting system use to include spatially localized biomarker release.

In conclusion, we have proved the principle, for the initial time to our knowledge, that targeted ultrasonic deposition of energy to tumors can amplify biomarker signals in the blood as well as identify the biomarker release site. Consequently, our research directly addresses the current limitations of biomarker work, which include the difficulty in separating biomarker signal from noise and the inability to localize spatially the source of biomarker signal. The technique we have described could ultimately prove useful in humans to identify biomarkers in a visible lesion by appropriate application of the ultrasonic energy and measurement of biomarker levels before and after insonication. In this way, future work using image-guided focused ultrasound to insonicate tumors should help to bring together the currently separate fields of in vitro diagnostics and in vivo imaging and provide an advance in the progress to personalized medicine.

Methods
Cell Culture. The colon cancer cell line LS174T (American Type Culture Collection) was cultured in DMEM: nutrient mix F-12 complete media (Gibco 11330) supplemented with 10% (vol/vol) FBS (Gibco 26140).

CEA Quantification. CEA concentrations in media or plasma samples were determined using an ELISA from United Biotech, Inc. (catalog no. CM-201). Because the concentration of CEA in the plasma of the mice was at the lower end of the provided standard curve (1.5–30 ng/mL) of the kit, the standard curve was modified by dilution of the lowest standard to allow a more reliable curve fit at the expected lower concentrations (0.047, 0.09, 0.19, 0.38, and 0.75 ng/mL). The sensitivity of this assay was 0.05 ng/mL.

Ultrasound Treatment of Cells. LS174T cells were seeded at 1.5 × 10^5 cells in 6-well tissue culture plates (BD Biosciences; 35–3046) and grown overnight in complete media forming a 100% confluent monolayer of cells. On the next day, the media were removed, the cells were rinsed with media, and fresh media were added to the cells immediately before the ultrasound exposure. The cells were exposed to ultrasound using a 21.5-mm transducer of the Sonitron 2000 from the bottom of the plate. Ultrasound coupling gel (Aquanonic 100; Parker Laboratories, Inc.) was used to ensure a good contact between the transducer and the plate, with the transducer flushed against the plate. Media samples were collected and analyzed for CEA concentration by the ELISA. Control and ultrasound-treated samples were run in parallel in separate plates. Cell death was determined using Trypan blue (Invitrogen Co-op). The media were removed, and 100 μL of Trypan blue was added to the well. Live cells exclude the high M, dye, and dead cells with disrupted cell membranes take in the dye and stain blue. The percentage of dead cells was determined by counting the cells using a microscope.

Ultrasound Treatment of Mouse Models. Female nude mice (nu/nu; Charles Rivers Laboratories, Inc.), aged 6 to 8 weeks old, were s.c. implanted in the right flank area with 10 × 10^6 LS174T cells in 0.1 mL of PBS, pH 7.4, using a 28-gauge 0.5-inch needle. The tumors were grown for approximately 2 weeks until the majority of the tumors were greater than 0.3 cm^3 in volume. The mice were anesthetized with 2% (vol/vol) isofluorane in oxygen and kept on a heating pad during the treatment. Ultrasound was applied using the 21.5-mm transducer (Sonitron 2000). Ultrasound coupling gel (Aquanonic 100) was used between the transducer and the skin. Submandibular blood samples (n = 34) were collected using plasma separator tubes (BD Biosciences, catalog no. 36–5958), immediately before (pretreatment) and after (posttreatment) the ultrasound treatment. The volume of blood collected was ~75 μL per sample. No more than 10% of the blood volume of the mouse was collected per week, and 200 μL of warm saline was given s.c. to compensate for the loss of fluid. The separated plasma was analyzed for CEA concentrations by the ELISA. The change between pre- and post-concentration levels was used for comparison of CEA increases.

Controls included ultrasound treatments with blood analysis on the mice before tumor implantation (control 1) and on the non–tumor-bearing region (control 2). The individual animal served as its own control. All animal protocols were carried out with the approval of the Stanford Administrative Panel on Laboratory Animal Care.

The tumors from the mice were excised a day after the experiment, processed, and paraffin-embedded. Sections cut through the center of the tumors were stained with hematoxylin (stains nuclei acids) and eosin (stains proteins) and immunostained for the apoptotic enzyme caspase-3 (Histo-Tec Laboratories). The tissue was frozen using the Axyzert 200M microscope (Carl Zeiss Microlimaging), and the percentage of area stained for caspase-3 compared with the total area (n = 5 fields per section) was determined using the Metamorph Imaging Series software, version 7.0.4 (Molecular Devices).

Statistical Analysis. Because of the difficulty in obtaining precise values at low-concentration levels, nonparametric statistical methods robust to measurement error were used. The Kruskal-Wallis test was used to determine if the concentration of CEA in the plasma of the mice was at the lower end of the provided standard curve (1.5–30 ng/mL) of the kit, the standard curve was modified by dilution of the lowest standard to allow a more reliable curve fit at the expected lower concentrations (0.047, 0.09, 0.19, 0.38, and 0.75 ng/mL). The sensitivity of this assay was 0.05 ng/mL.