Final Progress Report (Richard J. Price, PI): Targeted Delivery of Controlled-Release Nanoparticles to Brain Tumors Using Contrast Agent Microbubbles and High-Intensity Focused Ultrasound

Over the one year time course of the project, we have worked out the fabrication protocol for the ultrasound-activated microbubble-nanoparticle composite agent (MNCA), developed 2 separate C6 glioma tumor models for mice, demonstrated the ability of the MNCA to deposit nanoparticles in three tissues (subcutaneous, skeletal muscle, tumors) upon activation with ultrasound, and generated controlled-release poly-lactide-co-glycolide (PLGA) nanoparticles bearing 5-FU that will eventually be incorporated into a therapeutic version of the MNCA. Perhaps most importantly, we have used these preliminary results to secure a 3 year grant from the Hartwell Foundation to continue this work into the future.

After successfully generating the MNCAs, which are comprised of controlled-release nanoparticles bound to the protein shell of contrast agent microbubbles, we tested whether this formulation yielded greater nanoparticle delivery to tissue upon intravenous injection when compared to a protocol in which the same number of microbubbles and nanoparticles are simply co-injected. To this end, we used a well-established hindlimb skeletal muscle model from our lab and MNCAs with fluorescent tracer nanoparticles attached to the microbubble shell. The results from this study, shown in Figure 1, indicate that the MNCAs are indeed far better at depositing nanoparticles in ultrasound-exposed tissues than simply co-injecting the microbubbles and nanoparticles. This result is important because it provides justification for bonding the nanoparticles to the microbubbles. Another new result is the successful demonstration of nanoparticle delivery to subcutaneous dorsal skinfold window chamber tissue with the MNCA (Figure 2). As shown in Figure 2, after ultrasound application, petechiae were created in the tissue and “clouds” of nanoparticles (green fluorescence) were distributed into the tissue.

While developing the MNCA protocol and testing it in both skeletal muscle and subcutaneous tissue, we concurrently began developing two glioma mouse models that would eventually be required for testing the agent in vivo. In the first model, rat C6 glioma tumors were engrafted into dorsal skinfold window chambers implanted on Rag2 mice (Figure 3). As shown in Figure 3, these tumors did engraft and become vascularized in some cases, leading us at first to believe this would be a good foundation for further tests. Unfortunately, we found that the typical engraftment time in the windows was very long, presumably due to the lack of thermal regulation in the skinfold tissue which was physically elevated above the body. While other investigators have been able to work around this limitation, we eventually decided that this was not acceptable for our application. For this reason, we decided to switch from the

Figure 1. The composite delivery agent yields more efficient nanoparticle delivery than co-injection when delivered intravenously. **A:** Photomicrographs of skeletal muscle showing nanoparticle (green fluorescence) deposition following ultrasound application (top row) or no ultrasound application (bottom row). Note the enhanced nanoparticle delivery with the composite agent when compared to co-injection (yellow arrows). **B:** Bar graph of nanoparticle deposition showing significantly enhanced delivery with the composite agent. * $P < 0.05$.

Figure 2. **A:** Low magnification image showing window chamber surgically implanted on a mouse. **B:** Subcutaneous microcirculation as viewed through the window using intravital microscopy. **C-G:** Ultrasound application to dorsal skinfold window chamber following i.v. injection of microbubble-nanoparticle composite agent elicits petechiae creation and nanoparticle delivery. **C,D:** Low magnification images of window chamber tissue before (**C**) and after (**D**) ultrasound. Discrete petechiae are clearly present in **D**. **E,F:** Higher magnification images showing a region of window chamber tissue before (**E**) and after (**F**) ultrasound. Arrows denote petechiae. **G:** Region of window chamber tissue showing 100 nm fluorescent nanoparticle delivery. Arrows denote nanoparticle “cloud” in tissue. Chamber has a diameter of 1 cm.

Figure 3. Photomicrographs of a C6 glioma tumor implanted in the dorsal skinfold window chamber. **A:** Low magnification image showing circular tumor in center of window. **B:** High magnification inset showing dense and disorganized microvessel networks on the edge of the tumor.

window model to a more standard subcutaneous model. To this end, we injected C6 glioma cells subcutaneously into the mouse flank. The growth curve for the subcutaneous tumors is shown in Figure 4A. This model has proven to be very robust, so we are now designing our future pre-clinical therapeutic experiments around it. Indeed, as shown in Figure 4B, we have begun to deliver fluorescent nanoparticles to ultrasound-targeted subcutaneous C6 gliomas. In Figure 4B, a region of tumor tissue (labeled with red fluorescence using a quantum-dot tracker) with a small cloud of tracer nanoparticles (green fluorescence) is shown. Moving forward, while we are disappointed that we spent considerable time on the ineffective window chamber model, we have the flank model well in hand. We are now beginning to measure how the activation of the MNCA with ultrasound affects perfusion, tumor growth, and nanoparticle delivery in these tumors and will continue to do so with support from the Hartwell Foundation.

Finally, looking ahead to studies that will test the ability of the agent to deliver a chemotherapeutic agent, we are making PLGA nanoparticles containing the chemotherapeutic drug 5-FU. To verify that the 5-FU remains biologically active following its encapsulation in the polymer, we have performed cell viability assays using rat C6 glioma cells (Figure 5). The results from these studies have shown that the 5-FU nanoparticles do indeed reduce cell viability to a level that is comparable to that achieved with the soluble drug (positive control). Furthermore, the negative control studies indicate that the polymer alone has no influence on cell viability. Although we haven't optimized the release of the drug from the polymer, with this first-generation agent in hand, we are reasonably confident that we can generate proof-of-principle for our methodology.

Figure 4. **A:** Glioma growth curve following subcutaneous implantation. **B:** Photomicrographs of a glioma (red fluorescence) following ultrasound-mediated delivery of nanoparticles (green) from the composite agent.

Figure 5. Viability of C6 glioma cells in the presence of 5-FU loaded PLGA nanoparticles. PLGA nanoparticles containing 5-FU at both 4 and 8 µg/ml polymer generated significantly reduced cell viability, comparable to soluble 5-FU. * indicates significantly different than unloaded PLGA group ($P < 0.05$).