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Combined Vascular Targeted Imaging and Therapy: A Paradigm for Personalized Treatment

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Abstract In order to be successful in personalizing treatment, methods for selecting patients as well as good surrogate biomarkers for monitoring the effects of treatment are required in addition to development of an efficacious targeted therapy. We have developed a polymerized nanoparticle platform technology that will allow us to put different targeting moieties on the surface of the particles in addition to loading the particles with different contrast and therapeutic agents. We have proven that these nanoparticles can be targeted to endothelial receptors and different payloads of contrast and therapeutic agents have been delivered to target cells with high target to background ratios. Using this combined vascular targeted imaging and therapy approach, we are optimistic that personalized treatment regimens can be developed for different disease processes such as cancer, inflammation, and ischemia. J. Cell. Biochem. Suppl. 39: 65–71, 2002. © 2002 Wiley-Liss, Inc.

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In this post-genomic era, "personalized medicine" has become a popular term. However, "personalized medicine" requires the development of many clinical tools including risk algorithms based on understanding of the molecular basis of disease, molecular diagnostics for categorizing the patients, effective targeted molecular therapeutics, and surrogate biomarkers for monitoring the effect of the therapy. To be successful, all the necessary elements in the chain have to be developed in parallel right from the beginning [Ginsburg and McCarthy, 2001]. In this review, we summarize our results in designing a platform technology that allows us to deliver different therapeutic and diagnostic agents to activated endothelium using a variety of antibodies and ligands as the targeting moieties. Since the same drug delivery platform is used for both therapeutic and diagnostic agents, there is a built-in patient selection and treatment monitoring strategy co-developed with the treatment agent, thereby satisfying the requirements for "personalized treatment."

ENDOTHELIAL RECEPTORS AS MOLECULAR TARGETS

Research in the past couple of decades have identified unique molecules called cell adhesion molecules (CAMs) that are found to be upregulated by endothelial cells during a variety of physiological and disease processes. These endothelial ligands and receptors are critical for promotion of leukocyte adhesion to the vascular endothelium, which is a critical first step in any inflammatory or immune response [Simionescu and Simionescu, 1988; Stoolman, 1989; Osborn, 1990; Springer, 1990; Lasky, 1992; Stoolman, 1992]. Since these endothelial receptors are more accessible to molecules delivered via the blood stream, many of the physiologic barriers to the delivery of macromolecules to parenchymal targets can be bypassed. Years of immunoscintigraphy research shows that even

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in the ideal case only 0.00–0.01% of intravenously administered monoclonal antibodies will reach and bind to parenchymal targets in humans [Gohr-Rosenthal et al., 1993]. This is because a blood-borne molecule has to be distributed through the vascular space, transported across the vascular wall and the interstitial space before getting to the intended target. There are many opportunities for these molecules to bind non-specifically to proteins or other tissue components and/or be metabolized. Because of the significance and accessibility of endothelial receptors, they are attractive targets for molecular diagnostic and therapeutic agents.

POLYMERIZED NANOPARTICLES AS DELIVERY VEHICLE

Before we embarked on the development of a platform for combined vascular targeted imaging and therapeutics, we listed the ideal features that are required for the delivery platform to be successful: (a) biocompatible, (b) sufficiently long intravascular half-life to allow for repeated passage through and interactions with the activated endothelium, (c) ability to have ligands and proteins conjugated on the surface in multivalent configuration to increase the affinity and avidity of interactions with endothelial receptors, (d) ability to have functional groups for high-affinity surface metal chelation, (e) ability to carry drugs and nucleotides, and (f) capability to have both imaging and therapeutic agents loaded on the same vehicle. We were attracted to liposomes in the beginning since they have been extensively studied as drug delivery vehicles and stealth liposome encapsulated doxorubicin (Doxil) is now commercially available [Lasic et al., 1999; Brandl, 2001; Harrington, 2001; Park, 2002]. However, conventional liposomes are not very stable and it is difficult to chemically modify their surfaces. Polymers are easier to chemically modify but biocompatibility and configurations of the polymers are less desirable. So, we decided on developing a hybrid polymerized nanoparticle with desirable properties of liposomes as well as polymers. The polymerized nanoparticles that we chose to work on are composed of amphiphilic lipid molecules with polar head groups and hydrophobic tails that form aggregated bilayer type structures in aqueous solution [Storrs et al., 1995a,b]. A diacetylene group was incorporated into the tail portion of the lipids, which will cross-link upon irradiation with UV light polymerizing the lipids into structurally stable particles. Targeting moieties such as antibodies, ligands, contrast ions, and therapeutic agents for imaging and therapy can then be attached to the nanoparticles [Sipkins et al., 1998, 2000]. The size distribution and rigidity of these particles can be chosen to avoid rapid clearance by the reticuloendothelial system, and the particle surface can be modified with ethylene glycol or other molecules to further increase intravascular recirculation times [Storrs et al., 1995a,b].

DEVELOPMENT OF VASCULAR-TARGETED MRI CONTRAST AGENTS

Although MRI can provide exquisite morphologic details it is intrinsically a very insensitive technique. Since a large number of contrast ions (10^5–10^6) can be attached to each polymerized nanoparticle, the amplification of contrast effect by the particle is theoretically sufficient to allow for detection of endothelial receptors with MRI in vivo. With this approach, we can potentially combine the high spatial resolution provided by MRI with the sensitivity and specificity traditionally only available through the use of nuclear scintigraphy and positron emission tomography (PET). Towards this end, we have successfully synthesized and characterized paramagnetic polymerized nanoparticles (NPs) and have studied the use of these NPs as MRI contrast agents [Storrs et al., 1995a]. We began with the synthesis of a unique lipid containing pentacosadiynoic acid (PDA) conjugated to diethylene triamine pentaacetic acid (DTPA) via a variable length polyethylene glycol (PEG) linker. These amphipathic molecules have metal chelates as head groups connected to a lipid tail that contains a polymerizable diactylene moiety. By choosing different commercially available PEG derivatives, the linker length can be controlled. The PEG linkers on the surface of NPs also increase the recirculation of these particles in the blood pool by evasion of the RES. These NPs are 100–200 nm in diameter as determined by transmission electron microscopy (TEM). By extrusion of the suspension through polycarbonate filters with well defined porosity at temperatures greater that the T_m, the size of the NPs can be further controlled. After forming the NPs of the desired size by

extrusion, they are polymerized by cooling the solution to 4°C and irradiating the solution at 254 nm. For conjugating monoclonal antibodies to NPs, we constructed a particle containing biotinylated lipids. We then attach biotinylated antibodies to the NPs via an avidin bridge [Sipkins et al., 1998, 2000]. The resulting NPs are dark blue, with absorption bands at 544, 588, and 638 nm (λmax), but they turn red on gentle heating (absorption maxima at 498 and 538 nm).

MRI OF INTEGRIN UPREGULATION IN TUMOR NEOVASCULATURE

Vascular cell adhesion events are thought to play a significant role in angiogenesis [Folkman, 1992; Kohn and Liotta, 1995]. The adhesion receptor integrin $\alpha_v\beta_3$ has been identified as a marker of angiogenic vascular tissue and potentially can be used as a target for diagnostic and therapeutic agents aimed at diseases characterized by neovascularization such as cancer. A monoclonal antibody directed to $\alpha_v\beta_3$, LM609, has been demonstrated to promote tumor regression by inducing apoptosis of angiogenic blood vessels in human melanomas and breast cancers in SCID mouse/human chimeric models [Brooks et al., 1994, 1995]. We decided to incorporate LM609 as the targeting moiety on our NPs to be used for in vivo detection of tumor induced angiogenesis with MRI.

We used a rabbit V2 carcinoma model to test the hypothesis that LM609-labeled-NPs can be used to detect $\alpha_v\beta_3$ upregulation in tumor neovasculature. We inoculated V2 carcinoma cells into the thigh muscle or subcutaneously into the flanks of New Zealand white rabbits. In vivo MR imaging experiments were carried out when a palpable tumor (~1–3 cm) was established. Rabbits were injected with either 5 ml/kg LM609-labeled NPs (1 mg antibody/kg, 0.005 mmol Gd^{+3}/kg) or control NPs with isotype matched control antibodies. Coronal MR images were obtained immediately prior to contrast injection and at immediate, 30 min, 1 h, and 24 h post-contrast injection. Immediately following the imaging experiments, the tumor tissues were harvested for immunohistochemical studies. Figure 1 illustrates the MR findings of a V2 carcinoma carrying rabbit injected with LM609-labeled NPs. At immediate, 30 min and 1 h post-contrast injection there was no noticeable enhancement of the tumor or tumor margin as compared to the pre-contrast image (A). At 24 h post-contrast injection (B), there is obvious enhancement of the tumor margin. With the isotype control NPs little enhancement was observed even at 24 h post-contrast injection in both tumor models (compare images C to D in Figure 1). Figure 2 illustrates immunohistochemical findings in tissues obtained at the tumor margin of the same rabbit shown in Figure 1. Figure 2A was stained using anti-$\alpha_v\beta_3$ antibodies and Figure 2B was stained using polyclonal anti-mouse antibodies which should bind to the LM609 antibodies on the NPs. The stained regions in Figure 2A,B are identical showing that the LM609-labeled NPs have indeed co-localized with the sites of $\alpha_v\beta_3$ upregulation [Sipkins et al., 1998]. Also note that the NPs are localized to the vessels and not distributed throughout the tumor cells proving that the

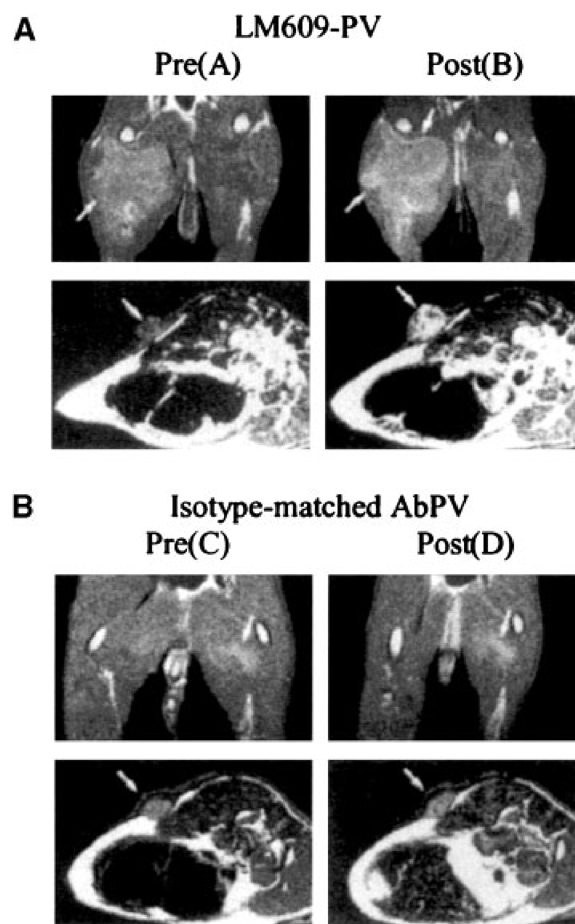

Fig. 1. **A**: MR images of V2 carcinoma in the thigh muscle of a rabbit and subcutaneously prior to (A), and at 24 h (B) post LM609-labeled ACPN injection. **B**: MR images of isotype matched controls (This figure is adapted from Fig. 1 of Reference 15).

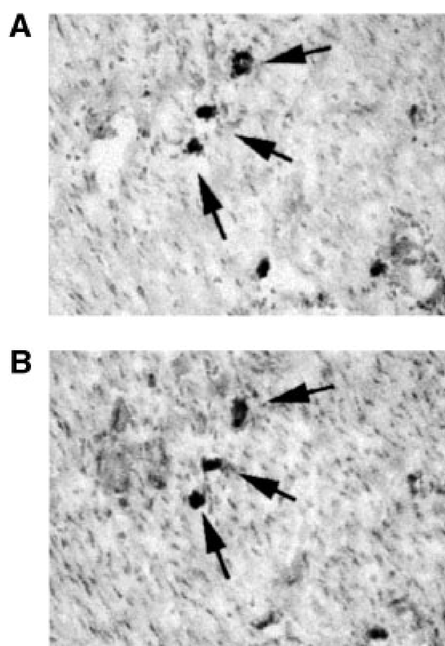

Fig. 2. IImmunohistochemical slides taken at the tumor margin of the same rabbit shown in Figure 1. Figure 2A was stained using anti-$\alpha_v\beta_3$ antibodies and Figure 2B was stained using polyclonal anti-mouse antibodies which should bind to the LM609 antibodies on the NPs. Arrows indicate the most prominently stained areas (This figure is adapted from Fig. 2 of Reference 15).

NPs did not localize to the tumors via passive accumulation.

IMMUNOSCINTIGRAPHY OF INTEGRIN UPREGULATION IN TUMOR NEOVASCULATURE

To prove that our platform technology can be used for different types of imaging detection, we have also investigated imaging of a solid tumor with LM609 labeled NPs with indium 111 (^{111}In) as the contrast ion [Bednarski et al., 2000]. LM609-^{111}In-NPs were injected intravenously into rabbits with Vx2 carcinoma in their thighs when the tumor reached a size of 3 cm^3. Scintigraphic imaging was performed over a 72-h period and showed accumulation of 22% of the total injected radiation in the tumor at 72 h. In rabbits injected with ^{111}In-NPs, which lacks the LM609 antibody, only 3% of the total injected radiation was found in the tumor at 72 h. The target-to-background ratio of the dose to the tumor vasculature is extremely high because of confinement of the antibody labeled NPs to the tumor vasculature, which represents a small fraction of the total tumor mass. The blood-pool half-life was 18 h for the LM609-^{111}In-NPs and was similar to the ^{111}In-NPs without antibody.

VASCULAR TARGETED GENE THERAPY USING POLYMERIZED NANOPARTICLES

Since the seminal work by Folkman and coworkers, there has been tremendous interest in using tumor vasculature as a primary target for antiangiogenic treatment [Folkman, 1975]. Many antiangiogenic treatment approaches have had limited success in clinical trials [Ellis et al., 2001]. Patient selection and inability to monitor drug delivery and responses at the molecular level may be contributory factors for these disappointing early results. To overcome these problems, we have developed a cationic form of a targeted nanoparticle (NP) that can bind tumor vasculature with high specificity, transport genetic material into the tumor endothelium, and be followed using molecular imaging techniques (Fig. 3). An integrin antagonist (IA) was synthesized that binds the integrin $\alpha_v\beta_3$ [Eliceiri and Cheresh, 2001] with high specificity as the targeting moiety. The IA-targeted NP was constructed as previously described [Hood et al., 2002]. A plasmid with the mutant form of *Raf-1* was used as the therapeutic agent. It has been previously shown that the disruption of the Ras-Raf-MEK-ERK pathway suppresses angiogenesis in vivo, and suppression of *Raf-1* activity has been reported to promote apoptosis [Felding-Habermann et al., 1992; Wickham et al., 1993]. The mutant form of *Raf-1*used in the gene therapy experiment fails to bind ATP (ATP$^{\mu}$-Raf), and has a dominant negative effect. To evaluate the therapeutic efficacy of this targeted NP/plasmid complex (IA-NP/Raf(−)), systemic injection of the complex in 6 mice bearing established 400 mm^3 M21-L tumors was performed. The M21-L tumors do not express the integrin $\alpha_v\beta_3$, so any observed therapeutic effect should be from the effect on vascular endothelial cells alone. Control groups received systemic injections of the non-targeted complex (nt-NP/Raf(−)), or PBS (six mice with established 400 mm^3 M21-L tumors per group). A blocking experiment was also performed with the co-injected of IA-NP/Raf(−) and a 20-fold molar excess of a soluble form of the IA. Our results demonstrated that control mice injected with PBS or nt-NP/Raf(−) formed large tumors ($>$1,200 mm^3) within 25 days and were euthanized. In contrast, mice

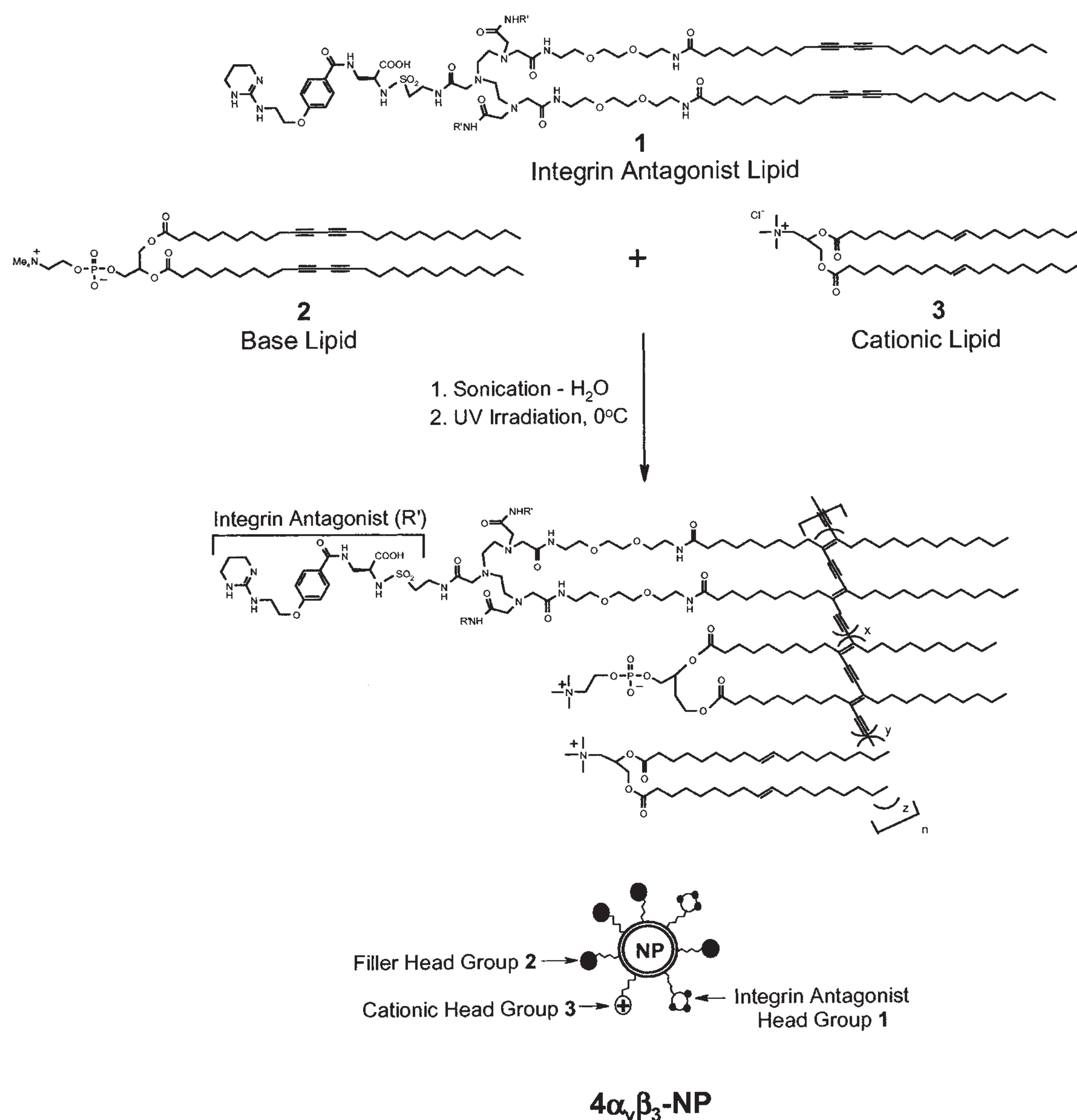

Fig. 3. Formation of targeted NPs for gene delivery to the tumor endothelium (This figure is reprinted with permission from Fig. 1 of Reference 28).

receiving a single injection of IA-NP/Raf(−) showed rapid tumor regression (Figs. 4 and 5), with four of the six mice showing no evidence of tumor 6 days post IA-NP/Raf(−) treatment. The two remaining mice showed a >95% reduction in tumor mass. It is important to note that the tumor regressions were sustained for >250 days, suggesting that the indirect effect of this approach may be widespread. It is also significant that injection of excess soluble IA $\alpha_v\beta_3$ ligand, completely blocked the anti-tumor effect of IA-NP/Raf(−). This demonstrates that this is a specific effect based on the ability of the IA-NP/plasmid complex to target the vasculature, achieve gene expression and promote apoptosis of the angiogenic endothelium [Hood et al., 2002].

We further evaluated the use of IA-NP/Raf(−) by using a syngeneic pulmonary and hepatic metastases mode 1 of colon carcinoma. Murine CT-26 carcinoma cells were either injected intravenously to induce pulmonary metastases or intrasplenically into Balb/C mice to induce hepatic metastases. NP/gene complexes were then given after these metastases were allowed to establish for 10 days. Control groups included

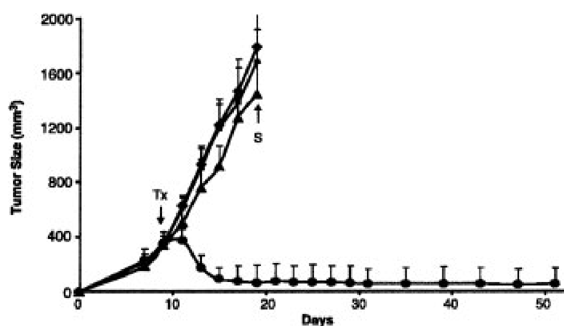

Fig. 4. Tumor size measurements as a function of time. ◆PBS, ●NP-Plasmid, ▲Blocking experiment, ■targeted NP-Raf(−) plasmid, Tx = treatment, S = sacrifice (This figure is adapted with permission from Fig. 4 of Reference 28).

mice treated with PBS, IA-NP complexed to a control vector, or a nt-NP/Raf(−). Our results showed that in the control mice, extensive tumor burden in their lungs or livers were observed, whereas in mice treated with IA-NP/Raf(−) little or no visible tumor metastases was present. This was quantified by a drastic reduction of the wet lung or liver weight. Blocking experiments in which mice received a co-injection of IA-NP/Raf(−) along with an excess of a soluble targeting ligand showed tumor burden similar to that in control mice [Hood et al., 2002].

CONCLUSION

Our results demonstrate that it is feasible to use polymerized nanoparticles as a platform to deliver imaging and therapeutic agents to activated endothelium. This opens up the possibility of using combined vascular targeted imaging and therapy for personalized treatment. Many different combinations of contrast agent, therapeutic agent, and targeting moieties can potentially be used. In this paradigm, vascular targeted imaging will be performed prior to initiation of therapy. Based on the results of the imaging test, only the patients that demonstrate adequate level of upregulation of the targeted endothelial receptors will receive the treatment. Following treatment, vascular targeted imaging can be used to monitor the effects and guide modulation of the therapy regimen. We are optimistic that with multidisciplinary teams developing the right combinations of molecular diagnostic, therapeutic agents and surrogate biomarkers, "personalized treatment" will be available for many disease processes in the future.

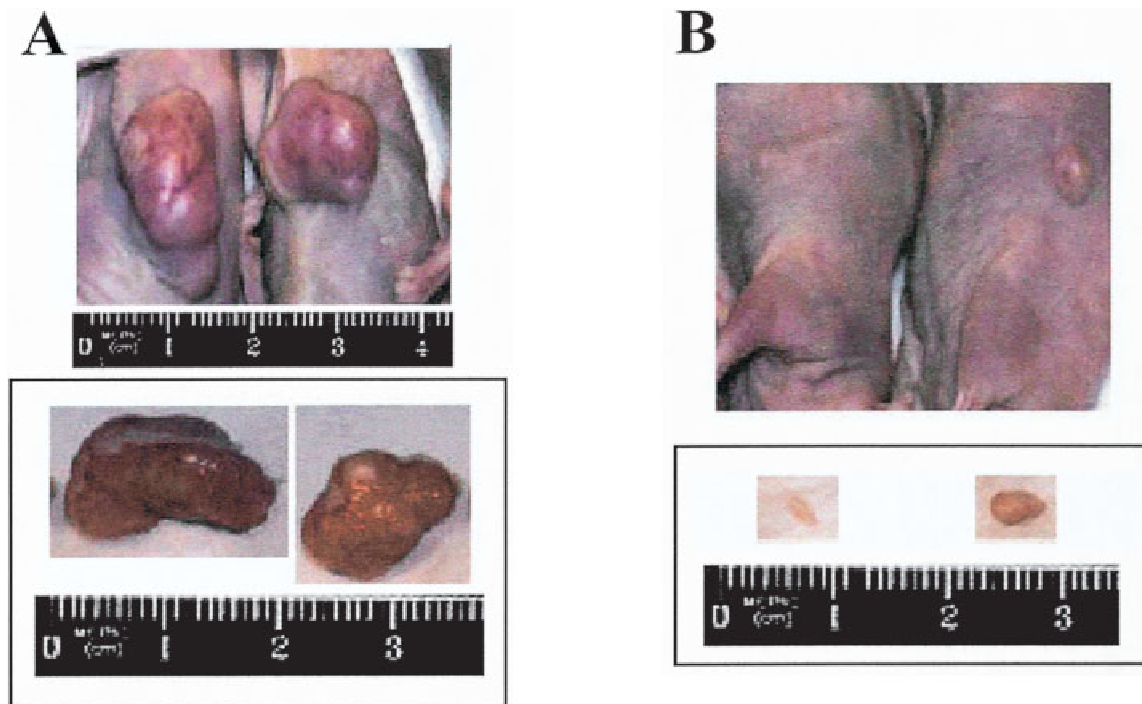

Fig. 5. M21-L murine melanoma tumors control animals (left), animals treated with a single injection of IA-NP-Raf(−) complex (right) (This figure is adapted with permission from Supplemental Fig. 2 of Reference 28).

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