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SHORT COMMUNICATION

Assessing the effect of a nude mouse model on nanoparticle-mediated gene delivery

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Abstract The relevance of using nude mouse models for evaluating drug delivery to human tumors has recently been questioned by numerous researchers. While the immune response is known to play a critical role in cancer, this study assesses the effect of using immunocompromised "nude" mice on drug delivery. By inoculating both nude and immunocompetent mice with a mouse mammary carcinoma cell line (4T1), differences in the "first pass effect", distribution, and reporter gene expression due to the use of the nude mouse model could be elucidated. Our results indicate that initial tumor deposition (5 min) was slightly lower in nude mice but comparable after 24 h. In addition, some small differences in tissue deposition/accumulation and reporter gene expression were observed between the two mouse models. The results with this one tumor model suggest that delivery studies conducted in nude mice can provide comparable results to those in immunocompetent mouse models.

Keywords Immunocompetent · Nude mice · Immunocompromised · Gene delivery · Lipoplex · Biodistribution

Introduction

The advent of nanotechnology offers the promise of overcoming technological hurdles in many fields, including drug delivery [1]. While nanoparticles composed of many different materials (e.g., lipids, proteins, polymers) are designed to deliver a variety of therapeutic cargos (e.g., small molecules, peptides, nucleic acids), the critical barriers to delivery (e.g., targeting, immunogenicity, toxicity, stability, clearance) represent obstacles for all of these systems [2]. Not surprisingly, many of the efforts for developing improved delivery systems continue to focus on targeting therapeutics to tumors to achieve more efficacious cancer treatment. With the desire to test these approaches on human tumors, the majority of studies have utilized immunocompromised "nude" mice as an in vivo xenograft model that can accommodate human cancer cells. Although this strategy has the advantage of allowing therapeutics to be tested on human tissue in an animal model, the wisdom of assessing delivery performance and efficacy in an immunocompromised setting has recently been questioned [2–4]. Furthermore, a recent study by Rios-Doria et al. clearly demonstrates that the immune system can play a significant role in the efficacy of traditional chemotherapeutics [5]. In addition to the role of the immune system in cancer treatment, resistance, and progression, it is conceivable that a compromised immune system could have an effect on delivery in terms of the biodistribution and tumor delivery [2].

Alternatives to assessing a therapeutic strategy on human tumors in a nude mouse model are to generate spontaneous tumors or utilize a mouse cancer cell line in a fully immunocompetent "normal" mouse. This has the advantage of testing efficacy and delivery in a more realistic setting in which the effects of the immune response are not compromised [3, 4]. However, this approach has the disadvantage of not allowing testing on human tumors. Because researchers employ immunocompromised mice in order to conduct experiments on human tumor cells, whereas immunocompetent mice must employ murine tumor cells, it is difficult to directly compare results and to understand the effects of using an immunocompromised mouse model on delivery and biodistribution. To

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investigate this issue, we implanted mouse mammary carcinoma cells (4T1) in both immunocompromised "nude" and immunocompetent Balb/c mice and assessed differences in tumor size, the first pass effect, biodistribution, and expression of a reporter gene delivered by a well-characterized, serum-stable lipoplex. This short communication summarizes our findings.

Methods

Materials

Cholesterol and N-(1-(2, 3-dioleoyloxy) propyl)-N,N,N-trimethylammonium chloride (DOTAP) were purchased from Avanti Polar Lipids (Alabaster, AL) and used to prepare liposomes at a DOTAP/cholesterol weight ratio of 31:69 as previously described [6–9]. Liposomes were then mixed with a luciferase-encoded plasmid [10] at a charge ratio of 4 [9]. The plasmid used in these experiments has been modified to reduce immunostimulatory CpG sequences and contains a ROSA26 promoter (CMV removed) that exhibits reduced but sustained in vivo expression [10, 11]. This lipoplex preparation has a particle size and zeta potential of 189 ± 17 and 55 ± 3, respectively, and these values change to 196 ± 14 and −47 ± 4, respectively, after serum exposure [10]. Lipoplexes were diluted 1:1 (*v*/*v*) with 12 % hydroxyl ethyl starch (MW 250,000, Fresenius; Linz, Austria) prior to administration, and 50 μg DNA was injected via a tail vein as previously described [9].

Prior to treatment with lipoplexes, female immunocompetent Balb/cJ (stock #000651) and athymic nude Nu/J (stock #002019) mice 6–10 weeks old were acquired from Jackson labs (Bar Harbor, ME) and inoculated in each shoulder with 4T1 murine mammary carcinoma cells (ATCC #CRL-2539). Nude mice were inoculated with 1×10^7 cells, and immunocompetent mice were inoculated with 1×10^6 cells as recommended in previous studies [12]. Tumor size was monitored with calipers, and luciferase expression was monitored in extracted tissues with Promega Luciferase Assay Reagents (Madison, WI) as previously described [9]. All institutional and national guidelines for the care and use of laboratory animals were followed.

Determination of plasmid levels in tissues

To determine delivery of plasmid DNA to tissues in the mouse models, animals were sacrificed 5 min and 24 h after intravenous administration of lipoplexes, and their organs were harvested and flash frozen in liquid nitrogen. Organs were then thawed and weighed, and DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD). Quantitative PCR (qPCR) was then performed on the samples using QuantiTect RT-PCR Kit (Qiagen, Germantown, MD) on an Applied Biosystems 7500 RTPCR instrument (Grand Island, NY). A standard curve of pure plasmid was used for quantification along with amplicon efficiency factors to account for amplification that is not perfectly efficient (as suggested by the Applied Biosystems 7500 Manual referencing Fenster et al. "Real-Time PCR." Current Protocols Essential Laboratory Techniques, 2009: 10.3.1–10.3.33). Plasmid levels (5 min) and luciferase expression (5 min and 24 h) in the two mouse models were compared by using a one-way ANOVA, and $p < 0.05$ was considered significant.

Extraction efficiency

In order to determine the extraction efficiency of the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD), a known amount of plasmid DNA was injected into the organs freshly harvested from Balb/c mice. Each organ was processed per the Qiagen DNeasy protocol and then measured by qPCR. A standard curve of pure plasmid was used, and extraction efficiencies were calculated based on the amount of plasmid recovered relative to the amount injected (50 μg). These efficiencies were used to adjust DNA recoveries in our experiments.

Results

After subcutaneous inoculation of mice with 4T1 cells, tumor size was monitored for 9 days prior to treatment with lipoplexes. As shown in Fig. 1, tumor size increased more rapidly in nude mice reaching 114.2 ± 6.3 mm^3 as compared to tumors in immunocompetent mice which grew to 47.9 ± 9.2 mm^3 in the same time period. It is important to point out that it is common to inoculate nude mice with higher numbers of tumor cells in order for all inoculated tumors to grow (see methods) [12–14], and this likely contributes to the more rapid tumor growth observed as compared to that seen in immunocompetent mice.

The initial tumor deposition and accumulation were assessed by monitoring the levels of plasmid in the tumor 5 min and 24 h, respectively, after intravenous administration. As shown in Fig. 2, plasmid levels in the tumor 24 h after injection were comparable in the two animal models and were similar to that seen 5 min after injection, suggesting that the initial deposition played a predominant role in tumor accumulation regardless of the animal model. Although the difference between animal models was statistically significant after 5 min, no difference in tumor accumulation was observed after 24 h. Considering the larger experimental variability in the 24 h measurements, it is possible that a slight difference (≈ 10 %) exists between the animal models, and further

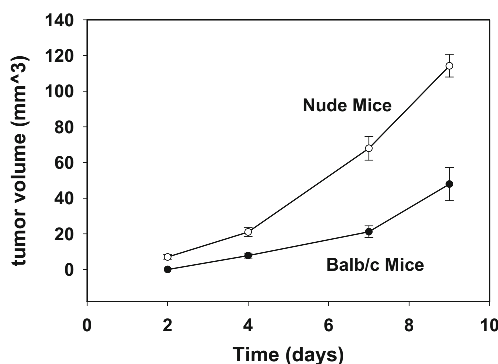

Fig. 1 Growth of 4T1 tumors in nude and immunocompetent mouse models, $n = 6$

experimentation would be required to determine if the small differences were statistically significant.

Plasmid levels in the liver, lung, spleen, kidney, and heart were also quantified at 5 min and 24 h, and similar trends were observed with regard to the lack of a significant difference between nude and immunocompetent animal models (Fig. 3). In general, the initial deposition after 5 min was similar to that observed at 24 h. Somewhat lower levels in the liver and kidney were seen at the later time point, suggesting that the rate of degradation and/or clearance in these organs is greater than the rate of any additional accumulation that may occur after the initial deposition. In contrast, plasmid levels in the spleen were elevated two- to threefold at 24 h (as compared to 5 min), suggesting that lipoplexes may be actively processed by this organ after the initial deposition.

We also monitored luciferase expression in the tumor after 24 h (Fig. 4); it should be recognized that the processes of transfection and expression take several hours, and thus, luciferase activity is not measurable in tissues extracted from mice only 5 min after intravenous administration. The data 24 h after injection show that reporter gene expression in tumors from immunocompetent and nude mice was not significantly

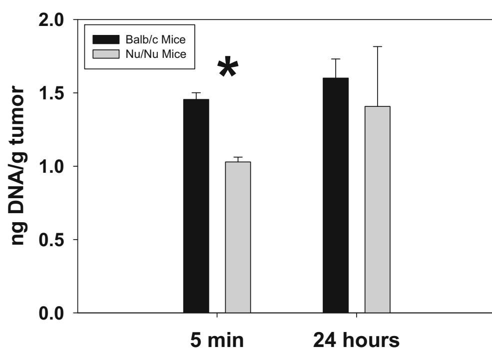

Fig. 2 Plasmid levels in tumors from immunocompetent (*black bars*) and nude mice (*gray bars*) at 5 min and 24 h. *Bars* represent the mean and one standard error from the three mice (two tumors/mouse). The *asterisk* indicates a statistically significant difference ($p < 0.05$) between the two animal models

different, consistent with the similar plasmid levels observed in tumors after 24 h (compare Figs. 2 and 4).

Similar to that observed in the tumor, expression after 24 h was comparable between the two animal models in the liver, spleen, and kidney, but significantly different expression was observed in the lung and heart (Fig. 5). Expression in the heart was consistent with the greater plasmid accumulation in this organ in immunocompetent mice at 24 h. Surprisingly, expression in the lungs was greater in nude mice despite the lower levels of plasmid accumulation (compare Figs 3b and 5). This discrepancy differs from our previous study where we observed a strong correlation between plasmid levels and expression after treating nude mice bearing KB tumors with different formulations [9]. The greater expression in the lungs of nude mice despite reduced plasmid levels (as compared to immunocompetent mice) suggests that some differences relevant to gene delivery may exist between these two tumor-bearing mouse models. Although it might be anticipated that differences would be observed between such different animal models (nude vs. immunocompetent), it is surprising that tumor accumulation and expression were comparable at 24 h.

Discussion

In considering our results, it is important to mention that tumor size is known to affect delivery, and thus, the different growth rates (Fig. 1) might obscure differences in tumor delivery between the two animal models. Indeed, studies investigating the effect of tumor size in animal models and in humans have concluded that smaller tumor size improves accumulation on a per gram basis [2, 15–17], and this effect might preferentially enhance delivery to the smaller tumors in the immunocompetent mouse model. While we cannot definitively rule out some contribution of tumor size, the fact that tumors had comparable plasmid levels and expression at 24 h suggests that any effect of tumor size in our study is minimal. In this regard, it is worth noting that even the largest of the tumors used in our experiments (i.e., from the nude mice) is sufficiently small (<0.2 g) to exhibit the enhanced delivery described in previous studies [15]. Our results could also be affected by differences in tumor vascularization and/or architecture which could potentially be related to numerous factors (e.g., genetic differences) in addition to immune status. Other limitations of our study are that only one dose of a single lipoplex formulation was administered, and therefore, the effects of different doses and/or formulation variables (e.g., size, charge, composition) could not be evaluated. While definitive conclusions would require that similar experiments be repeated in other mouse strains, bearing different tumors and with different delivery systems, our limited investigation suggests that delivery studies conducted in nude mouse models give comparable results

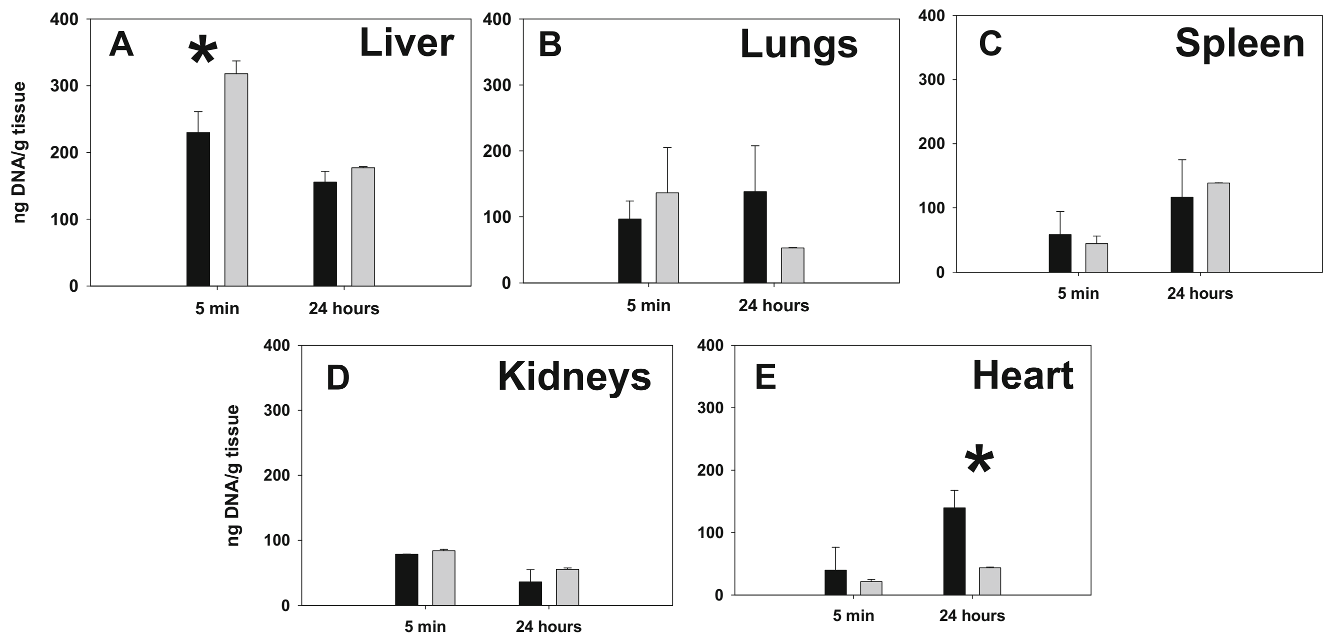

Fig. 3 Plasmid levels in organs from immunocompetent (*black bars*) and nude (*gray bars*) 5 min and 24 h. *Bars* represent the mean and one standard error from the three mice. The *asterisks* indicate a statistically significant difference ($p < 0.05$) between the two animal models

in terms of tumor delivery to that obtained in immunocompetent models.

Such a rapid distribution (i.e., within 5 min) is consistent with previous studies utilizing complexes of cationic lipid and DNA for intravenous gene delivery [18–22]. In many studies, the initial distribution occurs primarily in the lungs via embolism due to lipoplex-mediated aggregation of blood cells [18, 19, 23]. It has been shown that the lipoplexes that are entrapped in the lung via this mechanism are gradually released over time and ultimately accumulate in the liver [18, 23, 24]. In contrast, the lipoplex formulation employed in this study utilizes cholesterol as the helper lipid, and cholesterol-based formulations do not induce blood cell aggregation [25]. This is in agreement with our previous work demonstrating the enhanced stability of lipoplexes formulated at cholesterol levels sufficient to promote the formation of a cholesterol domain [6, 8, 26]. The fact that plasmid levels in the lung do not decrease dramatically between 5 min and 24 h, combined with the reduced plasmid levels in the liver after 24 h, suggests that lipoplexes do not initially distribute primarily to the lungs with subsequent redistribution to the liver. Instead, our data indicate a relatively uniform initial distribution to all organs as would be expected from a lipoplex formulation that does not induce blood cell aggregation. Furthermore, because stability in the blood is likely governed by the properties of the lipoplex, it is not surprising that distribution of a single dose of particles to naïve mice would not be altered by immune competence. However, it is well established that repetitive administration of PEGylated particles can elicit an adaptive immune response that profoundly alters distribution and clearance [27–31]. Although the lipoplexes used in this study do not contain PEGylated components, additional studies would

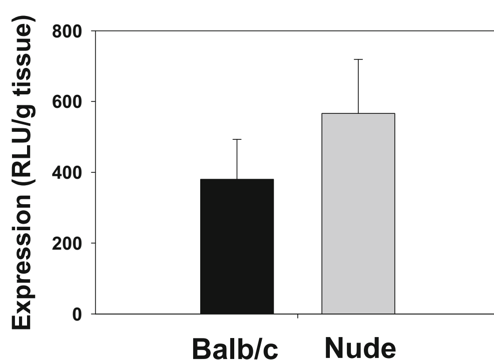

Fig. 4 Luciferase expression in tumors harvested from immunocompetent and nude mice 24 h after intravenous administration. *Bars* represent the mean and standard error from the three mice

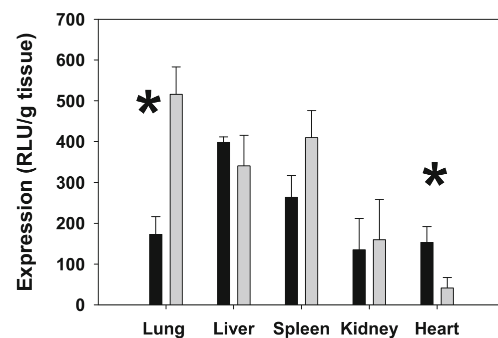

Fig. 5 Luciferase expression in organs harvested from immunocompetent (*dark bars*) and nude (*light bars*) mice. *Bars* represent the mean and standard error from the three mice. The *asterisks* indicate a statistically significant difference ($p < 0.05$) between the two animal models

need to be conducted to determine if immunocompetence of the animal model affects distribution upon repetitive administration.

In conclusion, the prevalence of utilizing nude mouse models with compromised immune function in order to assess delivery to human tumors in vivo has garnered increasing criticism as the fundamental role of the immune response in cancer becomes elucidated [2–4]. The goal of this study was to assess the effect of different mouse models (nude vs. immunocompetent) on drug delivery by utilizing the same tumor type (murine carcinoma) and delivery vehicle (lipoplex) in the two different strains of mice. Overall, we obtain comparable results for lipoplex distribution to the tumor in both animal models. Similarly, delivery to all tissues was comparable between nude and immunocompetent mouse models, although we did observe some differences in expression in the heart and lungs. Taken together, the data from this small study indicate that the initial deposition of lipoplexes at 5 min (i.e., "the first pass effect") and accumulation at 24 h is similar in the two mouse models and that the use of nude mice to assess tumor delivery does not artifactually elevate delivery to the tumor.

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Compliance with ethical standards All institutional and national guidelines for the care and use of laboratory animals were followed.

Conflict of interest The authors declare that they have no conflict of interest.

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