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Therapeutic efficacy of combined PEGylated liposomal doxorubicin and radiofrequency ablation: Comparing single and combined therapy in young and old mice

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ABSTRACT

Antitumor therapy in the elderly is particularly challenging due to multiple, often chronic diseases, poly-therapy, and age-related physiological changes that affect drug efficacy and safety. Furthermore, tumors may become more aggressive and drug-resistant with advanced age, leading to poor patient prognosis. In this study, we evaluated in mice bearing medulloblastoma xenografts the effect of age on tumor progression and tumor therapy. We focused on therapeutic efficacy of two treatment modalities alone radiofrequency ablation therapy (RFA), PEGylated liposomal doxorubicin (PLD) equivalent to Doxil, and their combination. We demonstrated that tumor growth rate was higher and survival was lower in old versus young mice ($p < 0.05$). Likewise, tumors in old mice were less susceptible to either PLD or RFA monotherapy. However, combined therapy of PLD and RFA succeeded to eliminate the age-related differences in anti-cancer treatment efficacy ($p > 0.05$) by the two monotherapies. The results on PLD therapy are supported by preferable PEGylated nano-liposomes accumulation in tumors of young mice compared to old mice, as determined by near-infrared imaging with indocyanine green (ICG)-labeled PEGylated nano-liposomes. Taken together, our findings suggest that age effects on tumor progression and tumor monotherapy outcome may potentially be related to changes in tumor microenvironment, and that these changes can be overcome by RFA as this technique abolishes these differences and significantly improves success of PLD treatment.

1. Introduction

Global improvement of health care and living conditions has led to an increase of the elderly population [1]. Along with aging comes increasing number and frequency of diseases, including cancer [2]. Antitumor therapy in advanced age is challenging because most patients have concomitant diseases and therefore use many medications. Aging is also associated with reduced intestinal motility, lower muscle mass, changes in total body water and fat content, and impaired hepatic and renal function, which lead to modified drug pharmacokinetics, in addition to potentially altered pharmacodynamics. The net effect of these changes on response to specific drugs is difficult to predict and needs be addressed on a drug-to-drug and individual-to-individual basis [3]. Such effect may be even larger for nano-drugs whose distribution is highly dependent on the tissue micro-environment. For example, potential changes in immune [4] and endocrine system [5] function may affect the cancer microenvironment and consequently cancer development and progression [6]. Hence, an improved understanding of the mechanisms responsible for age-dependent differences in cancer development and progression may lead to planning of more efficacious therapeutic regimens tailored to old people and to better design of nano-drugs for this population.

Studies performed in animal model systems and in humans [7] show that long circulating nano-liposomes selectively accumulate in tumors, thereby reducing the potential for systemic adverse effects [8]. The enhanced permeability and retention (EPR) effect mediates such nonspecific passive targeting to tumors [9]. Particles below 100 nm effectively extravasate trough leaky angiogenetic vessels [10]. The small size and PEGylation prevent the rapid uptake of these liposomes by reticuloendothelial system (RES), thus increasing their circulation time [10] and eventually improve the chances of successful therapy and reduce drug toxicity. A good example is Doxil®, PEGylated liposomal doxorubicin, which extends the circulating time of the active compound and has a

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"stealthy" behavior [7]. Doxorubicin is released from the liposomes only at the tumor's interstitial fluid due to the relatively high local concentration of ammonia, continuously produced there as a result of glutaminolysis (a tumor cells unique metabolic pathway) [11]. Thereafter, the released doxorubicin is selectively taken-up by the tumor cells and kills them. The effectiveness of treatment was shown to be augmented by radio-frequency ablation (RFA), a minimally invasive image-guided therapy [12–14]. The main limitations of RFA are the specific localization of its effect only to tumors to which it is applied and the lack of complete eradication of large tumors [15]. However, the RFA-modified tumor microenvironment leads to higher accumulation of doxorubicin at the tumor site [13] and induces apoptosis in the remaining viable cells [16], thus reducing tumor recurrence. The effectiveness of this combination was successfully demonstrated in focal mammary, liver and brain tumor models [17,18].

To date, little if any information is available on the effect of age on the therapeutic efficacy and toxicity of Doxil. However, based on the age-related changes in tumor micro-environment and the immune and endocrine system, it is likely that age would affect the outcomes of treatment with Doxil or its combination with RFA. Accordingly, this study aimed to revisit the age effect on tumor development and to evaluate the hypothesis that the success of treatment with Doxil is affected by age. To address this issue we treated nude mice bearing human medulloblastoma xenografts [19] with RFA and PLD. The model was chosen based on previous research demonstrating that doxorubicin and RFA combination is a potent, synergistic anticancer treatment for this type of tumor [20–22].

2. Material and methods

2.1. Material

Due to the shortage of commercial Doxil/Caeylx [23] because of FDA and EMA shut-down of its production site, we used DOX-NP™, (Avanti Polar Lipids Catalog number 300107S) a PEGylated liposomal doxorubicin very similar to Doxil® which is formulated by Lipocure Ltd. (Jerusalem, Israel), and sold by Avanti Polar Lipids (Alabaster, AL, USA). Indocyanine green (ICG) was from Acros (Geel, Belgium). All other reagents were purchased from Sigma-Aldrich (Rehovot, Israel).

2.2. Preparation of liposomes for the in vitro studies

Doxorubicin-free ("empty") liposomes were prepared as previously described [24], with minor changes. For this, lipid hydration was performed in solution of 10% sucrose in DDW. Two types of liposomes were produced by extrusion: PEGylated liposomes, with lipid composition: HSPC- cholesterol-PEG-DSPE, final mole ratio 57:38:5 (similar to Doxil® and to DOX-NP™) at total lipid concentration of 55 mM; and non-PEGylated liposomes, lacking DSPE-PEG having a lipid composition of: HSPC - cholesterol, mole ratio 57:38, and total lipid concentration of 52 mM. Both types of liposomes were prepared as multilamellar vesicles, PEGylated (MLV-PEG^{+}) and non-PEGylated (MLV-PEG^{-}). A fraction of these two types of MLVs were down-sized by extrusion to obtain two types of small unilamellar vesicles, PEGylated (SUV-PEG^{+}) and non-PEGylated (SUV-PEG^{-}) of the same lipid composition as the above MLVs. The size and polydispersity index (PDI) of these SUVs were similar to Doxil® [24] and DOX-NP.

2.3. Preparation of liposomes labeled with ICG

Aliquots of 3 mM ICG in DDW was added to 40 mg lipid/mL of SUV dispersion of similar composition to Doxil® and to DOX-NP, but lacking drug to a final ICG concentration of 500 μM. This dispersion was mixed overnight at 4 °C to form small unilamellar vesicles labeled by ICG, either PEGylated (SUV-ICG-PEG$^{+)}$ or non-PEGylated (SUV-ICG-PEG^{-}) [25].

2.4. In vitro studies

We endeavored to understand the in vivo fate of ICG when it is a part of Doxil-like PEGylated nano-liposomes (SUV-ICG-PEG^{+}). For this experiment, 50 μL of the donor liposomal ICG dispersion (SUV-ICG-PEG^{+} or SUV-ICG-PEG^{-}) at total lipid concentration of 55 mM, was mixed with 950 μL of the desired (ICG-free) either MLV or SUV dispersion. The lipid weight ratio of donor liposomes to acceptor liposome was 1:20. PEGylated (MLV-PEG^{+}) or (SUV-PEG^{+}) and non-PEGylated (MLV-PEG^{-}) or (SUV-PEG^{-}) were used as ICG acceptor liposomes. The samples were vortexed for 1 min and incubated at 4 °C under light protection and moderate mixing with a rotamixer (Rotamix RM1 from ELMI, Latvia). Samples were incubated for 0, 1, and 24 h. At the end of incubation, samples were centrifuged at 14 kRPM for 10 min using centrifuge Model 5417C, Eppendorf (Hamburg, Germany). The supernatant and pellet were collected. Under those conditions all MLV are in the pellet, while all SUV are in the supernatant. For analysis of ICG transfer between liposomes, the supernatant and the pellets were diluted 1:1 with ethanol (150 μL of each). The resulted dispersion was diluted 20 fold in DDW (50 μL of dispersion in 950 μL of DDW). 100 μL of the dispersion removed for fluorescence intensity measurements. The measurements were performed by Cytation 3 Cell Imaging Multi-Mode Reader from Biotek instruments (Winooski, VT), using an emission filter of 780 nm and excitation wavelength of 820 nm. Calculation of emission level supernatant and pellet were performed by using the following formulas:

$$\%\ \text{of total emission in supernatant} = \frac{\text{FLU (supernatant)}}{[\text{FLU (supernatant)}] + [\text{FLU (pellet)}]} \times 100\%$$

$$\%\ \text{of total emission in pellet} = \frac{\text{FLU (pellet)}}{[\text{FLU (supernatant)}] + [\text{FLU (pellet)}]} \times 100\%$$

FLU is ICG fluorescence emission intensity.

2.5. The in vivo animal model

Approval of the Institutional Animal Care and Use Committee of the Hebrew University (#MD-07-10404-5) was obtained before the initiation of these studies and experiments were conducted in accordance with the institutional guidelines. Studies were conducted in nude-Hsd:Athymic mice (Harlan Laboratories, Jerusalem, Israel), designated "young" (4 to 5 week old) or "old" (1.5 year old mice, corresponding to human age of 60 years [26]); mice were raised from the same batch of nude mice in the specific pathogen-free facility (SPF) unit at the Ein Kerem campus of the Hebrew University.

The number of mice used in the different experiments was dictated by the availability of old suitable mice.

The human medulloblastoma cell line (Daoy) was purchased from American Type Culture Collection (Manassas, VA). Approximately 4 million Daoy cells were inoculated s.c. to the back of the mice of each age group [27]. Direct caliper measurements were used for determination of tumor size [27–29]. Four to six weeks later, tumors grew to the desired diameter, 11 ± 2 mm for the survival study. For histopathologic study and the tumor size was 13 ± 2 mm for the evaluation of treatment effectiveness.

2.6. RFA application

Conventional monopolar RFA was applied by using a 500-kHz RFA generator (Model 3E; Radionics, Burlington, MA), as previously described [22]. Briefly, animals were placed on a standardized metallic grounding pad (Radionics), and electrode contact was made with the bare skin of the nude mice covered liberally with electrolytic contact gel. Initially, the 1 cm tip of a 21 gauge electrically insulated electrode (SMK electrode; Radionics) was located at the midpoint of the tumor. The RFA duration was 3 min, with the generator output titrated to