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Liposomal prednisolone inhibits tumor growth in a spontaneous mouse mammary carcinoma model

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ABSTRACT

Cancers are abundantly infiltrated by inflammatory cells that are modulated by tumor cells to secrete mediators fostering tumor cell survival and proliferation. Therefore, agents that interfere with inflammatory signaling molecules or specific immune cell populations have been investigated as anticancer drugs.

Corticosteroids are highly potent anti-inflammatory drugs, whose activity is intensified when targeted by nanocarrier systems. Liposome-targeted corticosteroids have been shown to inhibit tumor growth in different syngeneic murine tumor models as well as human xenograft mouse models, which is attributed to a switch in the tumor microenvironment from a pro-inflammatory to an anti-inflammatory state. Despite the recognized value of implantation tumor models in preclinical research, the "acute" inflammation induced by inoculation of tumor cells together with the exponential tumor growth in a relatively short period of time does not resemble slow progressive human disease that develops in situ. Therefore, in this study, the antitumor effect of liposomal corticosteroids was investigated in a clinically more relevant setting of transgenic mice developing spontaneous breast carcinomas.

Here we show that liposomal prednisolone phosphate inhibits the growth of spontaneous breast carcinoma. Interestingly, the liposomal prednisolone was significantly more active than free drug. At 72 h after injection of the liposomal formulation, 3 μg prednisolone per gram of tumor tissue was recovered whereas no drug could be recovered after injection of the free agent. This indicates that, despite etiological and morphological differences between implanted and spontaneous tumor models, EPR-mediated accumulation of drug occurs to similar extent in this spontaneous mammary carcinoma model as in the syngeneic tumor models.

Finally, we analyzed miRNA profiles in the MMTV/*neu* model and showed that the top 10 of miRNAs in the MMTV/*neu* tumor consisted of miRNAs with a known involvement in breast carcinoma proliferation and metastasis. The only exception was the appearance of miR-146b, a known inflammation-regulating miRNA species, after liposomal prednisolone treatment.

1. Introduction

Tumors are complex assemblies of multiple cell types in a net favorable environment for their survival, growth, invasion and dissemination. Cancer growth, invasion and metastasis appear largely dependent on the ability of the mutated malignant cells to hijack and exploit physiological processes of the host. A dysfunctional inflammatory response is one of the hallmarks of cancer [1–3]. Several malignancies arise at sites of chronic infection (e.g. Hepatitis B virus infection, *Helicobacter pylori* infection) and inflammation (autoimmune diseases, such as inflammatory bowel disease) [4]. But also after tumor initiation, the microenvironment of a developing tumor often harbors a large infiltrate of innate and adaptive immune cells and associated inflammatory mediators [5]. Whereas full activation of innate and especially adaptive immune cells may translate in eradication of the mutant cells, the chronic activation of inflammatory cells within the tumor microenvironment is known to support tumor proliferation, survival and migration. Activated inflammatory cells produce signaling mediators (cytokines, chemokines, growth factors), in general to ensure protection against injury and promote tissue homeostasis. In cancer, these processes seem to be co-opted by the tumor cells to foster cell survival and proliferation and reach an immune privileged status. This offers the

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possibility for therapeutic strategies that are aimed at interfering with these signaling molecules and specific immune cell-subtypes to modulate -and ultimately shift- the inflammatory microenvironment towards an anti-tumor phenotype [6,7].

One of the promising classes of compounds to achieve such a shift are steroidal anti-inflammatory drugs [8]. They possess strong anti-inflammatory and immunomodulatory activities that translate in anti-tumor effects in vitro and in vivo. Various genomic and non-genomic mechanisms of action could mediate this therapeutic effect. Genomic effects already seem to take place at low concentrations of corticosteroids, while non-genomic mechanisms require higher concentrations. A combination of both types of effects appears to be necessary as antitumor activities are achieved only at very high doses. These concomitantly can lead to the occurrence of severe adverse effects inherent to the strong systemic immunosuppression, which can even lead to death due to opportunistic infections [9].

The therapeutic index of corticosteroids and their anti-inflammatory effects can be substantially increased by incorporation of corticosteroids in nanocarrier-platforms such as polymeric micelles and liposomes [10–13]. The leaky architecture of the tumor vasculature tissues allows the passive delivery of long-circulating nanomedicines to the tumor tissue by the "enhanced permeability and retention (EPR) effect" but also macrophage-rich organs like liver, spleen and bone marrow are targeted, which could also be involved in the therapeutic effect [14]. The distribution of the drug to other sites is limited, reducing the occurrence of certain side effects.

The antitumor activity of liposomal corticosteroids has been studied in different subcutaneous murine tumor models, particularly in B16F10 melanoma and C26 colon carcinoma [15]. In both experimental models, a single intravenous administration of prednisolone encapsulated in long-circulating liposomes (LCL-PLP) inhibited tumor growth in a dose-dependent manner.

By definition, animal models are an approximation of human disease. However, spontaneous tumor models are likely to be closer to the clinical situation than the transplantation models that we have used thus far [16,17]. The acute injection of a mass of ex vivo cultured tumor cells may contain a different inflammatory milieu than spontaneous tumors. A frequent concern in developing new anti-cancer drugs is the difficulty to predict drug activity in human disease when employing murine tumor models. Thus, more advanced, genetically engineered mouse tumor models may better predict the ultimate clinical activity of drug molecules, since such models display orthotopic primary tumors in an immune competent setting [18].

In this study, we investigated the accumulation in the tumor and antitumor effects of LCL-PLP in transgenic mice that carry the unactivated *neu* (Erbb2) oncogene under the control of the mouse mammary tumor virus (MMTV). This mouse model is characterized by the spontaneous development and slow growth of breast cancer over a period of months [19].

2. Materials and methods

2.1. Liposome preparation

Liposomes were prepared as described previously [12]. In brief, appropriate amounts of dipalmitoylphosphatidylcholine (Lipoid GmbH, Ludwigshafen am Rhein, Germany), cholesterol (Sigma-Aldrich, Germany), and poly (ethylene glycol) 2000-distearoylphosphatidylethanolamine (Lipoid GmbH), in a molar ratio of 1.85:1.0:0.15 respectively, were dissolved in ethanol in a round-bottom flask. A lipid film was prepared under reduced pressure on a rotary evaporator and dried under a stream of nitrogen until complete dryness. Liposomes were prepared by rehydration of the lipid film with a solution of 100 mg/ml prednisolone disodium phosphate (BUFA, The Netherlands). Liposome size was reduced by multiple extrusion steps (Lipex high pressure extruder, Northern Lipids) using polycarbonate membranes (Whatman, Nuclepore) with a final pore size of 50 nm. Mean particle size of the liposomes was determined by dynamic light scattering with a Malvern ALV CGS-3 system and found to be 0.1 μm in a monodisperse system. Total lipid content of the liposomal dispersion was determined with a phosphate assay on the organic phase after extraction of liposomal preparations with chloroform and according to Rouser et al. [20]. Liposomal dispersion was transferred to a Slide-A-Lyzer cassette (Thermo Scientific, Waltham, MA, USA) with a molecular weight cut-off of 10 kD in order to remove unencapsulated drug by dialysis at 4 °C with repeated changes of buffer. The aqueous phase after chloroform extraction was used for quantification of prednisolone phosphate by Ultra Performance Liquid Chromatography (UPLC, Waters Acquity UPLC- TUV system, Waters Corporation, Milford, MA, USA). Measurements were performed using an Acquity BEH C18 1.7 μm column (2.1 × 50 mm, Waters) and the mobile phase consisted of acetonitrile (Biosolve, Valkenswaard, The Netherlands) and water (25:75 (v/v)), brought to pH 2 with perchloric acid (Mallinckrodt Chemicals, Chesterfield, UK). Detection was performed by a diode array detector set at a wavelength of 254 nm. The liposomal preparation contained approximately 5 mg of prednisolone/ml and 60 μmol lipid/ml. The liposome suspension was stored at 4 °C.

2.2. Inhibition of cell proliferation

MCF-7 cells (ATCC HTB-22) and MDA-MB-231 (ATCC HTB-26) human mammary carcinoma cells were incubated with PLP, LCL-PLP or liposomes as control (C). Cells were cultured in DMEM/F12 medium (Life Technologies Europe B.V., Bleiswijk, The Netherlands) containing 1.2 g/L sodium bicarbonate, 3.6 g/L HEPES, 3.2 g/L D-glucose, 2.5 mM L-glutamine, and supplemented with 10% FBS. 10^3 cells/well were plated in a 96-well plate for 24 h. Subsequently, liposomal PLP and free PLP or vehicle were added in the respective wells. The anti-proliferative effect was determined over 48 h, by ELISA BrdU-colorimetric immunoassay (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. This technique is based on the incorporation of the pyridine analogue bromodeoxyuridine (BrdU) instead of thymidine into the DNA of proliferating cells. To detect BrdU incorporated in newly synthesized cellular DNA, a monoclonal antibody conjugated with peroxidase was added. After 90 min of incubation, cell lysates were washed three times with PBS. The immune complexes were detected by adding the substrate of peroxidase (tetramethyl-benzidine). The reaction product was quantified by measuring the absorbance at 450 nm with a reference wavelength of 655 nm.

2.3. In vivo studies

Transgenic female mice FVB/N-Tg (MMTV/*neu*) 202Mul/J (12–13 weeks age) were purchased from Jackson Laboratory (USA). Mice were kept in standard housing on a 12 h light/dark cycle with standard rodent chow and water available ad libitum. Experiments were performed in accordance to the national regulations and were approved by the local animal experiments ethical committee. Transgenic mice developed mammary tumors spontaneously within 3 to 7 months upon arrival, as described earlier [19]. Tumors were measured daily with a digital calliper and the tumor volume was calculated according to the formula: $V = 1/6\pi a^2 b$, where a is the smallest and b the largest superficial diameter. In a small number of mice, tumor growth was extremely fast going from palpable to over 200 mm^3 within 1 week. These mice were excluded as they deviate from the tumor growth curve that has been described for this model. Mouse weight was recorded in order to monitor weight loss as a result of toxic side effects. To evaluate the therapeutic effects of corticosteroids in a spontaneous tumor model, transgenic mice received 20 mg/kg of free or liposomal prednisolone phosphate or an equivalent volume of vehicle, intravenously via the tail vein when mammary tumors reached a size of 200 mm^3. The dose