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A novel liposomal Clodronate depletes tumor-associated macrophages in primary and metastatic melanoma: Anti-angiogenic and anti-tumor effects

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ABSTRACT

The depletion of tumor-associated macrophages (TAMs), involved in different stages of cancer development and progression, is an appealing strategy in cancer therapy.
We developed novel Clodronate-containing liposomes (Clo-Lipo-DOTAP) presenting physicochemical properties (size distribution, polydispersity index and Z-potential) suited for safe storage and injections.
In vitro, Clo-Lipo-DOTAP inhibited proliferation, reduced viability and induced apoptosis of a macrophage-like cell line in a dose- and time-dependent manner.
In proof of functionality experiments, Clo-Lipo-DOTAP depleted macrophages in a genetic mouse model of chronic hepatitis and hepatocellular carcinoma leading to a significant reduction of F4/80-positive cells in the liver and spleen of treated mice compared to PBS-treated controls. The number of granulocytes, B and T lymphocytes was not affected.
In B16/F10 subcutaneous melanoma-bearing mice, Clo-Lipo-DOTAP significantly reduced the volume of primary tumors ($P < 0.001$). Within the tumors, the expression F4/80 and α-SMA was significantly lowered. Plasma levels of IL-10, Mo KC, TNF-α, VEGF and PDGF-bb were statistically decreased. In B16/F10 lung metastatic melanoma model, treatment with Clo-Lipo-DOTAP significantly reduced the number of pulmonary nodules ($P < 0.05$). F4/80-positive cells and microvessel density were statistically decreased.
In conclusion, the depletion of TAMs in primary and metastatic melanoma presents anti-tumor efficacy *via* inhibition of angiogenesis and modulation of inflammation related cytokines.

1. Introduction

The tumor microenvironment (TME) is a complex system that plays a critical role in cancer development, progression and control. It consists of proliferating cancer cells, tumor stroma, blood and lymphatics vessels and infiltrating cells of the immune system [1]. Non-malignant cells of the TME are recruited from surrounding tissues and are "educated" by the transformed cells to acquire a pro-tumor phenotype. The dominant portion of infiltrating immune cells is composed by macrophages, defined as tumor-associated macrophages (TAMs) [2]. They derive from circulating monocytes, enrolled at tumor site by chemotactic factors.

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TAMs attempt to restore the normal function of damaged tissue, but their interaction with neoplastic cells in the TME modifies their properties, which results in immunosuppression and promotion of tumor growth [3]. Several studies have indicated that TAMs have an M2-like phenotype and they are characterized by pro-tumoral properties (*i.e.* induction of cell proliferation, angiogenesis and extracellular matrix turnover; inhibition of adaptive immunity) [4]. Furthermore, TAMs are closely associated partners of malignant cells for migration, invasion and metastasis formation [5].

TAMs are abundant in established tumors and their presence is associated with increased tumor progression and invasion. Furthermore, there are pre-clinical and clinical evidences that a prevalence of TAMs within tumors is associated with worse overall prognosis in a high proportion of solid tumors [6,7], emphasizing the importance of their interaction with neoplastic cells and suggesting that they may represent a promising target for novel anti-cancer strategies [8]. Since TAMs are involved in different stages of cancer development and progression, a therapy against macrophages can be adjusted so that it targets specific functional features, such as activation, recruitment and pro-angiogenic properties.

For a macrophage-targeted therapy, liposomes are, at present, the most investigated and appealing delivery systems, as they have various advantages, such as low immunogenicity, good biocompatibility, cell specificity and drug protection [9]. Furthermore, they are composed of materials easily functionalized, a feature that renders them extremely versatile tools. The inherent liposome properties also confer a natural targeting capacity for cells of the mononuclear phagocyte system [10].

Clodronate, currently used in the treatment of osteoporosis and bone metastasis, belongs to the drug family of bisphosphonates. In patients with advanced cancer, these agents have led to a great reduction in skeletal related events. Large, multilamellar liposomes containing Clodronate have been developed many years ago to specifically deplete macrophages [11]. Indeed, these Clodronate-liposomes, due to their big size, are rapidly recognized and taken-up by macrophages; subsequently, due to intracellular drug accumulation, macrophages undergo cell death via apoptosis [11]. In this regard, we and others have already demonstrated that Clodronate-containing liposomes are efficient in depleting macrophages in different disease models [12,13].

Here we developed a novel Clodronate-containing liposomal formulation, Clo-Lipo-DOTAP, with improved physicochemical properties, and investigated its functionality in depleting macrophages in an inflammation-driven model of carcinogenesis, and its anti-tumor effectiveness in biologically and clinically relevant murine melanoma models, known to correlate with infiltrating TAMs [14].

2. Materials and methods

2.1. Cell lines and animals

RAW 264.7 cells (murine, macrophage-like, cell line; American Type Culture Collection, Manassas, VA), B16/F10 cells (murine melanoma; kind gift of Dr. M.P. Colombo, Istituto Nazionale Tumori, Milan, Italy) and human skin fibroblasts from healthy donors (FIBRO/293) were used. All cells were grown in complete medium (Dulbecco's modified Eagle Medium; Euroclone S.p.A., Milan, Italy) supplemented with 10% fetal bovine serum (FBS, Euroclone S.p.A. Milano, Italy or Sigma-Aldrich, St. Louis, MO), 50 IU/mL sodium penicillin G, 50 μg/mL streptomycin sulfate and 2 mM L-glutamine (Sigma-Aldrich). B16/F10 cells were also stably transduced with a retrovirus for the constitutive expression of the firefly luciferase gene, as described [15], providing the B16/F10-luc cell line. B16/F10-luc cells were grown in RPMI-1640 medium (Euroclone) supplemented with 10% FBS, 50 IU/mL sodium penicillin G, 50 μg/mL streptomycin sulfate and 2 mM L-glutamine.

Cell lines were tested for mycoplasma contamination, cell proliferation and morphology evaluation, both after thawing and within four passages of culture.

C57BL/6JOlaHsd mice were purchased from Harlan Laboratories (Harlan Italy, S. Pietro al Natisone, Italy) and were housed under pathogen-free conditions, in Genoa at the Animal Facility of the IRCCS Azienda Ospedaliera Universitaria San Martino-IST Istituto Nazionale per la Ricerca sul Cancro; NEMO LPC-KO and NEMO FL mice have been described before [16,17] and were housed under similar conditions in the mouse facility at the Institute for Genetics, University of Cologne. Briefly, the ablation of NEMO in liver parenchymal cells causes spontaneous liver damage and leads to the development of hepatitis, fibrosis, hepatocyte dysplasia and ultimately hepatocellular carcinoma. Mice carrying loxP-site-flanked (floxed [FL]) NEMO alleles (NEMO FL) have been previously described [16]. NEMO FL mice were crossed to Alfp-Cre transgenic mice to generate a liver parenchymal specific knockout of this gene [NEMO LPC-KO; [17]]. In all experiments, littermates carrying the respective loxP-flanked allele, but lacking of the expression of Cre recombinase were used as controls (NEMO FL).

The *in vivo* experiments were reviewed and approved by the licensing and ethical committee of IRCCS Azienda Ospedaliera Universitaria San Martino-IST Istituto Nazionale per la Ricerca sul Cancro (Genoa, Italy) and by the Italian Ministry of Health. The work described was carried out in accordance with EU Directive 2010/63/EU for animal experiments http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm.

2.2. Development and synthesis of new Clodronate-containing liposomes (Clo-Lipo-DOTAP)

Clodronate (Sigma-Aldrich) was encapsulated into liposomes through a procedure based on the use of the cationic lipid DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate), introduced to improve the trapping efficiency of the drug, and DSPE-PEG_{2000} (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000], added to enhance the stability of the formulation.

Lipids (hydrogenated soy phosphatidylcholine (HSPC), cholesterol, DOTAP and DSPE-PEG_{2000}; Avanti Polar Lipids; Alabaster, AL, USA) were dissolved in Chloroform (final concentration 10 mM). Clodronate was dissolved in phosphate buffered saline (PBS) pH 9.5 (final concentration 150 mg/mL).

Briefly, DOTAP (3.2 mL) and Clodronate (1 mL) were mixed to promote an initial interaction. Subsequently, HSPC (8 mL), cholesterol (5.3 mL) and DSPE-PEG_{2000} (530 μL) were added to the DOTAP/Clodronate mixture (lipid molar ratio HSPC:cholesterol:DSPE-PEG_{2000}:DOTAP, 1.5:1:0.1:0.6). This was then sonicated (5 × 50 s), at a power of 50 Hz, with a probe sonicator (XL 2020 Sonicator; Misonix Inc., NY, USA) until the formation of a white emulsion, which was then transferred into a 50 mL glass ball and roto-evaporated under vacuum and a stream of nitrogen, by the use of a rotary evaporator (Laborota 4000, Heidolph Instruments, Germany) until the formation of a reverse phase. Liposomes were then repeatedly extruded through polycarbonate membrane filters (Merck Millipore, Darmstadt, Germany) of either 1 μm cutoff or of decreasing cutoff till 200 nm, thanks to a LiposoFast extruder (Avestin, Inc., Ottawa, ON, Canada). Liposomes sized at 1 μm and called 1 μm-Clo-Lipo-DOTAP were destined to deplete tissue resident macrophages, while the ones sized at 200 nm (200 nm-Clo-Lipo-DOTAP) were devoted to the depletion of tumor associated macrophages (TAMs).

Free Clodronate was then removed from Clo-Lipo-DOTAP either by centrifugation (30 min at 28,000 × *g*), in the case of 1 μm sized liposomes, or by passing them on a Sephadex G50 chromatography column, in the case of 200 nm sized liposomes.

Clodronate concentration was determined by the use of the van Rooijen method [11].

Particle size hydrophobic diameter (in nm), Z-potential (in mV) and polydispersity index (PdI) of Clo-Lipo-DOTAP were determined by dynamic light scattering, using the particle size analyzer Zetasizer