

Targeting Tumor Associated Macrophages Using Clodronate-loaded PLGA Nanoparticles

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ABSTRACT

It is now widely accepted that tumor associated macrophages (TAMs) are vital for tumor growth. In this study, we propose a novel strategy against cancer by targeting TAMs using drug-loaded nanoparticles. PLGA NPs containing clodronate, a bisphosphonate compound, were prepared by w/o/w emulsion method. Clodronate-loaded NPs showed a significant inhibitory effect on macrophage-like RAW264.7 cells while showing low toxicity towards CCL-110 human fibroblast cell line. Intravenously injected NPs functionalized with a TAM-targeting LyP-1 peptide also showed preferential accumulation in 4T1 tumor bearing mice. Our preliminary results suggest that clodronate-loaded PLGA NPs functionalized with the LyP-1 peptide can be potential anti-tumor agents.

Keywords: tumor associated macrophages, drug-delivery, clodronate, nanoparticles, LyP-1 peptide.

1 INTRODUCTION

It is now widely accepted that tumor associated macrophages (TAMs) are vital for tumor growth [1, 2]. TAMs originate from circulating monocytes and their recruitment into tumors is driven by tumor-derived chemotactic factors [3-5]. TAMs promote tumor cell proliferation and metastasis by secreting a wide range of growth and proangiogenic factors [6]. Consequently, many (but not all) tumors with a high number of TAMs have an increased tumor growth rate, local proliferation and distant metastasis. In fact, the extent of TAM infiltration has been used as an inverse prognostic predictor in breast cancer [7], head and neck cancer [8], prostate [9] and uterine cancer [9, 10]. TAMs are also prominent in tumor tissues, comprising up to 80% of the cell mass in breast carcinoma [11].

In recent studies, researchers have shown that anti-TAM effects induced by small molecule inhibitors leads to tumor suppression [6, 11]. For example, the antineoplastic agent Yondelis, having selective cytotoxicity towards TAMs,

significantly reduced the production of IL-6 and CCL2 by TAMs, which, in turn, contributed to growth suppression of inflammation-associated human tumors [12]. In another example, a bisphosphonate compound, zoledronic acid that suppresses MMP-9 secretion by TAMs was shown to inhibit the growth of cervical carcinomas [13]. In a different experimental model, inhibition of CCL5, a chemokine that facilitates the recruitment of TAMs in tumors, led to reduction in tumor infiltrate and slowed tumor growth [14]. Although therapeutic targeting of TAMs is still in its infancy, the above reasons highlight the importance of TAMs as a validated therapeutic target for cancer therapy and suggest that targeting TAMs may complement more conventional cancer treatment regimens [9, 10].

Clodronate is a bisphosphonate (BP) compound that can be used to selectively induce TAM apoptosis. BPs are used in the clinic to prevent or inhibit the development of bone metastases or excessive bone resorption and for the therapy of inflammatory diseases such as rheumatoid arthritis and osteoarthritis [15, 16]. Once delivered inside the cell cytoplasm BPs reacts with ATP generating methylene containing ATP analogs thereby inhibit cellular metabolism and inducing apoptosis [17]. Due to their high hydrophilicity and charge, BPs cannot easily penetrate the cell membrane and thus their effect on non-phagocytic cells is minimal.

Recent reports in media about results from studies conducted by two separate groups reported that women taking oral bisphosphonates developed about a third fewer breast cancers than other women [18]. Another independent study reported that clodronate improved the overall survival rate in men with metastatic prostate cancer [19]. BPs, therefore, are emerging as a promising drug for tumor therapy. In vivo, depletion of TAMs using clodronate-encapsulated liposomes has been shown to inhibit tumor growth in mouse models of cervical cancer [20], prostate cancer [21], teratocarcinoma and rhabdomyosarcoma [22], and in the inhibition of bone and muscle metastases of lung cancer cells [23]. However, since these liposomal formulations were not targeted they suffer from a serious side-effect of depleting circulating monocytes and other tissue associated macrophages such as

those found in liver, kidney and spleen [22]. This limits the prolonged administration of the drug which in turn leads to a repopulation of tumor stroma with macrophages in 1-3 weeks [20, 22]. Therefore, in order to use clodronate as an efficient anti-tumor drug it is imperative to minimize non-specific uptake of clodronate carriers by the RES macrophages.

Nanoparticles (NPs) provide the ideal platform to target and deliver bisphosphonates to TAMs. Interest in NPs for drug delivery and imaging has expanded exponentially over the past ten years (for reviews see Refs [24-26]). NPs, due to their small size, can pass through epithelial and vascular barriers and at the same time also present a large surface for functionalization with targeting moieties to achieve targeted drug delivery. Although many polymers can be used to fabricate NPs, only a few polymers have been approved for clinical use. Among these is poly(lactide-co-glycolide) (PLGA), a biodegradable and biocompatible copolymer which is used in a host of FDA approved therapeutic devices. The rapid escape of PLGA nanoparticles from the endo-lysosomal compartment into cytoplasm has been demonstrated, suggesting the suitability of PLGA NPs nanoparticles as a drug delivery vector [27]. Since the drug is encapsulated inside the polymeric matrix, it is protected from extracellular degradation. PLGA nanoparticles have been widely used as drug delivery vehicles to deliver small molecules, such as therapeutic agents, targeting moieties, imaging agents, amino acid residues or other organic moieties.

The large surface area of NPs and the availability functional groups on their surface provides an opportunity to functionalize them with a targeting ligand for receptors expressed on TAMs. LyP-1 is a candidate ligand for targeting tumor associated macrophages. LyP-1 is a cyclic nine-amino acid peptide (CGNKRTRGC) and a protein, known as p32 or gC1qR receptor, was identified as the target receptor for the LyP-1 peptide in tumors [28, 29]. The p32 protein is expressed both on tumor cells and TAMs in hypoxic/metabolically deprived areas of tumors. Quantum dots and abraxane nanoparticles coated with the LyP-1 peptide have been shown to specifically target tumor cells and lymphatic vessels upon intravenous injection in mice bearing tumors [30]. LyP-1-coated iron oxide nanoparticles also extravasate into the tumor and specifically bind p32-expressing cells [31].

The aim of this study is to develop a novel anti-macrophage agent that can be selectively targeted to TAMs. Towards this end, we report the fabrication of clodronate-loaded NPs made from PLGA polymer and its in-vitro efficacy in selectively inducing macrophage apoptosis. We also show preliminary results of LyP-1-functionalized PLGA NPs targeted to tumors.

2 MATERIALS AND METHODS

2.1 Materials

Clodronate was purchased from Sigma. Sulfo-SMCC was from Pierce. Biodegradable PLGA (50:50 lactide:glycolide, MW 47,000) was purchased from Boehringer Ingelheim. The organic solvent dichloromethane (DCM) and poly(vinylalcohol) (PVA, MW 9000-10,000, 80% hydrolyzed) were also purchased from Sigma.

Peptides were synthesized with automatic microwaveassisted peptide synthesizer (Liberty; CEM, Matthews) using standard solid-phase Fmoc/t-Bu chemistry. During synthesis, the peptides were labeled with 5(6)-carboxyfluorescein (FAM) with a 6-aminohexanoic acid spacer separating the dye from sequence. The synthesis of LyP-1 peptide used for the chemoselective ligation described with an extra N-terminal cysteine will be described elsewhere.

2.2 Preparation of nanoparticles

A water-in-oil-in-water double-emulsion-solvent-evaporation method was used to prepare clodronate-loaded PLGA NPs [32]. Briefly, 0.5 ml of a 4% aqueous solution of clodronate (pH adjusted to 7.4) was emulsified with 3 ml of 3% PLGA solution in DCM using a probe sonicator at 50W power for 60s. The primary emulsion was then added to 20 ml of 2% PVA solution containing CaCl₂ as 2:1 molar ratio of Ca:clodronate and emulsified at 50W power for 90s using a probe sonicator. DCM was evaporated by magnetic stirring for 4h at 4°C. Particles were collected by centrifugation and washed 3x with DDW, lyophilized and stored at 4°C until further use.

Amine-modified PLGA polymer for LyP-1 conjugation was prepared by conjugating hexaethylene glycol-diamine to the carboxylic terminal group of PLGA-COOH using the protocols as described in Yoon *et. al.* [33]. Amine-functionalized PEGylated PLGA nanoparticles were then prepared by using the double-emulsion-solvent-evaporation method as described above and using a 50:50 blend of PLGA-PEG and PLGA-NH₂.

LyP-1-functionalized PLGA NPs were prepared by coupling the LyP-1 peptide to amine-functionalized PLGA NPs through the peptide cysteine sulfhydryl group using a sulfo-SMCC cross-linker. Briefly, 20 mg NPs were suspended in 700 µl of PBS and 300 µl from 10 mM sulfo-SMCC was added. After incubation at room temperature for 30 minutes, the excess cross-linker was removed by filtration through a Nap-10 column (GE Healthcare). LyP-1 peptide labeled with FAM was dissolved in sterile nitrogen-purged water and added to the PLGA-sulfo-SMCC conjugate in small aliquots over a period of 1 hour. The peptide was used at twofold molar excess relative to PLGA. Excess peptide was removed by repeated centrifugation. Control NPs were prepared similarly except that LyP-1 was replaced with FAM-X-Cys (X refers to 6-aminohexanoic acid spacer).

2.3 Cell lines and tumors

The RAW264.7 monocyte/macrophages mouse cell line was obtained from the American Type Culture Collection (ATCC). They were cultured in DMEM media containing L-glutamine and sodium pyruvate (Mediatech), supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin/streptomycin. 4T1 cells were maintained in Dulbecco's modified eagle's medium supplemented with 10% FBS and 1% glutamine penicillin-streptomycin at 37°C and 5% CO₂. To induce 4T1 tumors, female Balb/c mice were injected with 2×10⁶ 4T1 cells in PBS into the mammary fat pad region. Two weeks after tumor cell injection LyP-1-NPs and control NPs were injected (15 mg/kg animal weight in 200 μl PBS) via the tail vein. The mice were sacrificed 3 hours post injection by cardiac perfusion under anesthesia, and organs were dissected and analyzed for particle homing. Tissues were processed and immunohistochemical analysis was performed as described previously [30].

3 RESULTS AND DISCUSSION

3.1 Clodronate NP formulation

These studies examined encapsulation efficiency (EE), morphology, particle size distribution and release kinetics of clodronate-loaded PLGA nanoparticles. During the study, morphology of the nanoparticles was studied through scanning electron microscopy. As seen from the electron micrographs in Fig. 1, the nanoparticles were smooth and spherical in shape. The size distribution illustrated in Fig. 2 shows an average diameter of 180 ± 20nm.

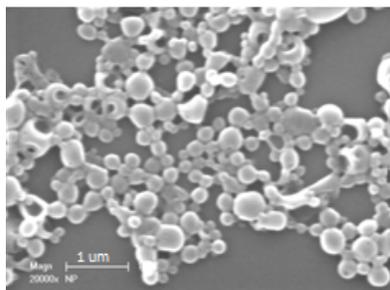


Figure 1: SEM image of clodronate loaded NPs made from PLGA polymer

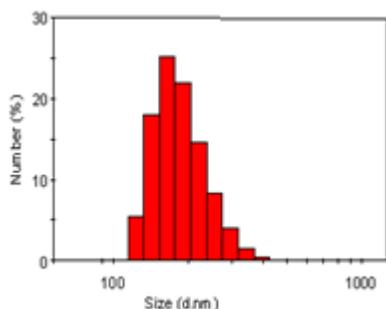


Figure 2: Dynamic Light Scattering (DLS) histogram showing size distribution of clodronate-loaded NPs

An analysis of the amount of clodronate encapsulated in the NPs revealed that 51% of the drug was entrapped in the particles. The release rate of entrapped clodronate was studied and the result is shown in Fig. 3. The kinetic data showed that majority of the drug release occurred in the first day (approximately 80%) and the release amount almost remained unchanged after 48 h. The “burst effect” release phenomenon, where drug is released in the very early stages, was observed as almost 50% of the drug was released in 4h. This release phenomenon is typical for a hydrophilic drug such as clodronate encapsulated using the w/o/w emulsion technique due the rapid migration of the water soluble drug towards the external aqueous phase.

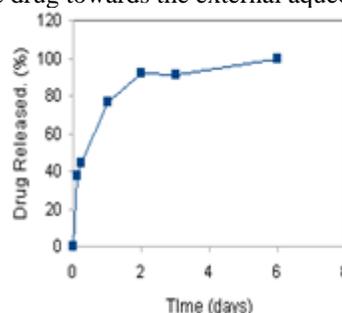


Figure 3: The drug release kinetics of clodronate-loaded NPs in vitro

3.2 Selective toxicity of clodronate NPs

We first tested the cytotoxicity of clodronate and clodronate-loaded PLGA NPs (clodNP) on mouse macrophage (RAW264.7) and human fibroblast (CCL-110) cell lines *in-vitro*. Cells were incubated with clodNPs as well as with blank NPs and free clodronate drug at a concentration of 250 μg/ml. Treatment with clodNPs resulted in an 8-fold increase in cell death compared to blankNPs and free drug. clodNPs showed little cytotoxicity towards human fibroblasts cell line (CCL-110) used as control (Fig. 4)

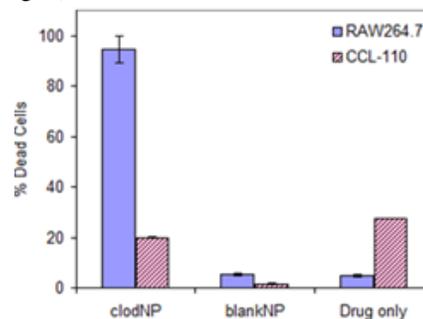


Figure 4: Selective abrogation of RAW264.7 macrophages by clodNPs compared to normal human fibroblast cells (CCL-110).

3.3 Tumor targeting with LyP-1-NPs

To test the efficacy of the LyP-1-NP conjugates for tumor “homing”, we injected them into Balb/C mice bearing 4T1 tumors. Our preliminary results show greatly enhanced accumulation of LyP-1-NPs in extravascular tumor tissue compared with control NPs (Fig. 8).

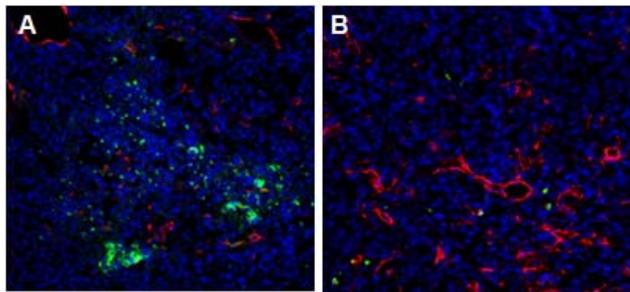


Figure 5: Targeting 4T1 tumors using functionalized NPs. (A) LyP-1-functionalized PLGA NPs show preferential accumulation in 4T1 tumors compared to (B) non-functionalized control NPs. NPs are stained green; cell nuclei are stained blue; CD31+ endothelial cells are stained red.

In summary, we have developed a novel clodronate delivery system to tumors using NPs made from FDA approved PLGA polymers and functionalized with a TAM-targeting peptide. Tumor therapy experiments are currently underway to investigate the effect of these NPs on TAM abrogation and tumor suppression.

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