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Research paper

Gellan gum nanohydrogel containing anti-inflammatory and anti-cancer drugs: a multi-drug delivery system for a combination therapy in cancer treatment

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ABSTRACT

During the last decades, it has become evident that inflammation plays a critical role in tumorigenesis: tumor microenvironment is largely orchestrated by inflammatory cells. In the present work, a novel gellan gum nanohydrogel system (NH) able to carry and deliver simultaneously anti-cancer and anti-inflammatory drugs was developed. Prednisolone was chemically linked to the carboxylic groups of gellan gum to serve as a hydrophobic moiety promoting nanohydrogel formation, whereas paclitaxel was then physically entrapped in it. NH improved drug performances, acting as paclitaxel and prednisolone solubility enhancer and favoring the drug uptake in the cells. Moreover, NH allowed an increased cytotoxic effect *in vitro* on several types of cancer cells due to the synergistic effect of the combination of anti-inflammatory and anti-cancer drugs. Thus, NH can be useful in a combination therapy that attacks both, malignant cells and tumor inflammatory components.

1. Introduction

The combination of anti-inflammatory and anti-cancer drugs is very promising for cancer therapy because inflammation can affect many aspects of tumor development and progression as well as the response toward the therapy. Clear evidence has been obtained that an inflammatory microenvironment is an essential component of all tumors, including some in which a direct causal relationship with inflammation is not proven yet [1,2]. Inflammatory microenvironment can promote the tumor initiation by increasing mutation rates [3] and affect tumor promotion. The inflammatory response may also have a role in the metastasis: tumor cells use the same adhesion molecules, chemokines and receptors to aid in migration and homing during distant metastatic spread [4]. These connections between cancer and inflammation lay the foundations for the use of anti-inflammatory drugs in cancer therapy. However, anti-inflammatory therapy is not cytocidal on its own and needs to be combined with more conventional therapies that kill the cancer cells.

In light of these findings, a multi-drug delivery system able to carry and release simultaneously anti-cancer and anti-inflammatory drugs in the required site could be very promising in cancer treatment for a combination therapy that attacks both, malignant cells and tumor inflammatory components.

In the current work, a recently developed nanohydrogel (NH) system [5] was used to deliver multiple drugs: prednisolone and paclitaxel. Prednisolone, a poorly water soluble anti-inflammatory drug [6], was chemically conjugated to the carboxylic groups of gellan gum and served as the hydrophobic moiety responsible for the self-assembly process. Gellan gum NH containing prednisolone has a core–shell structure that allows to hydrophobic drugs as paclitaxel (PCT) to be physically entrapped in its hydrophobic core. PCT disrupts the dynamic equilibrium within the microtubule system, thereby inhibiting cell replication. It has a very low water solubility and clinically it is used as a solution in Cremophor EL/ethanol (1/1, w/w), even though Cremophor EL causes many side effects, such as hypersensitivity, nephrotoxicity and neurotoxicity [7,8].

Gellan gum NH containing prednisolone and paclitaxel could be very useful for the treatment of inflammatory carcinoma or for several types of prostate cancer, where the combination of PCT and steroids is already used and allows to obtain several advantages. Prednisolone can reduce the side effects of the chemotherapy and act on cancer cells slowing their cellular growth. Combining this action with the toxic activity of paclitaxel, a synergic and more toxic effect on several types of cancer cells can be obtained.

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Glucocorticoids are in fact often included in the primary combination chemotherapy of inflammatory carcinoma such as lymphocytic leukemia, Hodgkin's and non-Hodgkin's lymphomas, multiple myeloma and breast cancer [9]. Moreover, the combination steroids and PCT is also used in the treatment of hormone refractory prostate cancer because of its ability to shrink the cancer and stop it growing for same time [10,11].

Hence, gellan gum NH able to target the drugs (PCT and prednisolone) in the tumor or its associated inflammatory area can be useful to combine the activities of the drugs, leading to superior therapeutic benefits.

2. Materials

Gellan gum was kindly provided by Fidia Advanced Biopolymers, Abano Terme (PD), Italy. Prednisolone, Paclitaxel (PCT) and Lipopolysaccharides (E. Coli 0111:B4) (LPS) were purchased from Sigma–Aldrich. Mouse TNF Elisa Kit was acquired from BD OptEIA. μBCA assay kit was obtained from Pierce (Rockford, IL). Cell Lysis Buffer was purchased from BD Pharmingen TM. CellTiter-Blue reagent Cell Viability Assay was acquired from Promega (Madison, WI). NIH/3T3 cells (murine fibroblast cells), H9C2 cells (murine myocardiocyte cells), PC-3 cells (human prostate cancer cells), A2780 (human ovarian cancer cells), MDA-MB-231 (human breast cancer cells) and Skov-3 (human ovarian cancer cells) were purchased from American Type Culture Collection (ATCC, Manassas, VA). NIH/3T3 cells, A2780 cells, MDA-MB-231 cells and H9C2 cells were grown in a humidified atmosphere of 5% CO2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin mixture (100.0 U/ml, 100.0 mg/ml, respectively). PC-3 cells were grown at 37 °C under 5% CO_2 in F-12K Medium supplemented with FBS and penicillin–streptomycin mixture. Skov-3 cells were grown at 37 °C under 5% CO_2 in RPMI Medium 1640 supplemented with FBS and penicillin–streptomycin mixture. Cell culture media and penicillin/streptomycin stock solutions were purchased from Cellgro (Herndon, VA). Heat-inactivated fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA).

3. Methods

3.1. Preparation of nanohydrogel with prednisolone

Prednisolone was chemically conjugated to the carboxylic groups of gellan gum, as described in [5]. Briefly, gellan gum molecular weight was reduced to 1.70×10^5 by ultrasounds treatment and 1.0 g of the polymer obtained was then dissolved in N-methyl-2-pyrrolidone (NMP) (135.0 ml). Prednisolone was previously derivatized by means of a four carbon atom chain (57.4 mg) and then dissolved in 39.0 ml of NMP. The two solutions were mixed and the reaction was kept under magnetic stirring for 40 h at 38 °C. An exhaustive dialysis against distilled water (Visking tubing, cut-off: 12,000–14,000) was then performed and the product Ge-Pred was finally recovered by lyophilization. In a typical preparation, 2.0 mg of the polymer obtained (Ge-Pred) was suspended in 1.0 ml of filtered distilled water and sonicated for 30 min in an ultrasonic bath sonicator (Strasonic-35, Liarre) to form the nanohydrogel (NH). The gellan-cholesterol (Ge-Chol) product was obtained by dissolving 1.0 g of sonicated gellan gum in N-methyl-2-pyrrolidone (NMP, 135.0 ml) and by adding cholesterol (60.3 mg), previously derivatized by a four carbon atom chain, dissolved in NMP (39.0 ml). The reaction was kept under magnetic stirring for 40 h at 38 °C. An exhaustive dialysis against distilled water (Visking tubing, cut-off: 12,000–14,000) was then performed, before recovering the product Ge-Chol by lyophilization.

Ge-Chol NH was prepared following the same procedure of Ge-Pred NH. In Ge-Chol polymer the hydrophobic moiety responsible for the self assembly process was cholesterol, instead the drug prednisolone used in Ge-Pred polymer.

3.2. Nanohydrogel characterization

The particle size and zeta potential of the formulations were measured using a Zeta plus Particle Analyzer (Brookhaven Instrument Corps, Santa Barbara, CA). Scattered light was detected at 25 °C at an angle of 90°. Samples (100.0 μl) of NH were diluted in 1.0 ml of distilled water and measured after the preparation.

3.3. Cytotoxicity of nanohydrogel

The cytotoxicity of the nanohydrogel (NH) was studied in NIH/3T3, H9C2 and PC-3 cells. The cells were seeded at a density of 5.0×10^3 cells per well into 96-well plates, 24 h before the experiment. Increasing concentrations of NH up to 1.0 mg/ml were incubated with cells for two different times of incubation: 4 h and 24 h. The cytotoxicity was evaluated by using the CellTiter-Blue reagent and was calculated with regard to the untreated cell control, which was set to 100% viability [12]. The assay was realized on Ge-Pred NH, on Ge-Chol NH, used as control, and on Triton X-100, used as positive control of cytotoxicity [13].

3.4. Nanohydrogel uptake and endocytosis inhibition

NIH/3T3 cells (10^5 cells/well) were seeded into 12-well plates and then cultured for 48 h. The cells were pretreated for 50 min at 4 °C, with low temperature as acting as inhibitor of endocytosis [14–16]. A further incubation (for 4 h) with the samples (prednisolone 250.0 μM in aqueous solution and in Ge-Pred NH, respectively) was also carried out at low temperature. The cells were then washed with fresh medium, with ice-cold PBS (phosphate buffer solution, pH 7.4), twice, and digested with trypsin. The lysis of the cells in the suspension was carried out by a lysis buffer to release the intracellular prednisolone. The resultant cell lysate was lyophilized and reconstituted with distilled water. The amount of prednisolone in the solution was measured using HPLC chromatographic system (HITACHI, EliteLaChrom), equipped with UV–Visible detector, an auto-injector system and a C18 column (4.6 mm × 250 mm). The mobile phase consisted of a mixture of acetonitrile and water in a ratio of 45:55. The flow rate was 1.0 ml/min and the injection volume 50.0 μl. The amount of prednisolone released was quantified by converting the UV absorbance at 242 nm against the standard calibration curve. The amount of prednisolone was then normalized to the total protein amount, determined by using μ-BCA assay kit [17–19].

The same experiment was repeated at 37 °C, without inhibiting the endocytosis, as control.

3.5. Intracellular kinetics of prednisolone release

NIH/3T3 cells (10^5 cells/well) were seeded into 12-well plates and then cultured for 48 h. The cells were treated with prednisolone (250.0 μM), in aqueous solution and in Ge-Pred NH, respectively, and then incubated for 1 h before washing twice with fresh medium.

After several times (30 min, 1 h, 2 h and 24 h) post incubation, cells were washed twice with ice-cold PBS and digested with trypsin. The lysis of the cells in the suspension was carried out by a lysis buffer to release the intracellular prednisolone. The resultant cell lysate was lyophilized and then reconstituted with distilled water. The amount of prednisolone in the solution was measured