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Efficient peritoneal dissemination treatment obtained by an immunostimulatory phosphorothioate-type CpG DNA/cationic liposome complex in mice

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Abstract

Peritoneal dissemination remains the most difficult type of metastasis to treat, and current systemic chemotherapy or radiotherapy tends to have little effect; therefore, immunotherapy using immunostimulatory CpG DNA could be a promising new therapeutic approach. Recently, we have reported that intraperitoneal administration of phosphodiester (PO) CpG DNA-lipoplex could efficiently inhibit peritoneal dissemination in mice. In this study, chemically modified phosphorothioate (PS)-CpG DNA and natural PO-CpG DNA were complexed with DOTMA/cholesterol cationic liposomes (PS-CpG DNA-lipoplex and PO-CpG DNA-lipoplex) and their antitumor activity was evaluated in a mouse model of peritoneal dissemination. Intraperitoneal administration of the PS-CpG DNA-lipoplex inhibited the proliferation of tumor cells in the greater omentum and the mesentery more efficiently than PO-CpG DNA-lipoplex. PS-CpG DNA-lipoplex induced higher cytokine production from primary cultured mouse peritoneal macrophages, suggesting that the high antitumor activity of the PS-CpG DNA-lipoplex is mediated by a high rate of cytokine production from immunocompetent cells such as macrophages. The serum transaminase levels of mice receiving intraperitoneal PS-CpG DNA-lipoplex treatment were measured to evaluate systemic toxicity, and these were found to be the same as those of untreated mice. These results suggest that intraperitoneal administration of PS-CpG DNA-lipoplex could be efficient immunotherapy for peritoneal dissemination.

Keywords: CpG DNA; Phosphorothioate; Peritoneal dissemination; Cationic liposome; Macrophage

1. Introduction

Peritoneal dissemination remains the most difficult type of metastasis to treat, and current systemic chemotherapy or radiotherapy tends to have little effect. The therapeutic problem of peritoneal dissemination is trans-lymphatic metastasis, that is, migration of the peritoneal free cancer cells through the lymphatic tissue, such as the milky spot on the greater omentum, spreading metastases throughout the body [1]. Since there are many immunocompetent cells in the lymphatic tissue, activation of these cells would be a new therapeutic strategy for peritoneal dissemination. It has been reported that CpG DNA, about 30-base oligonucleotides containing CpG dinucleotides, which is derived from the bacterial DNA, is recognized by Toll-like receptors (TLR)-9 expressed by macrophages and dendritic cells and induces Th1 type antitumor cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-12 [2,3]. Therefore, delivery of CpG DNA into lymphatic immunocompetent cells via the peritoneal cavity and induction of Th1 type cytokines in lymphatic tissues would effectively inhibit peritoneal dissemination.

As far as the molecular weight of the intraperitoneally injected compounds is concerned, a value of over 50,000 is required for efficient lymphatic organ distribution [4]. Since the molecular weight of naked CpG DNA is about 8000, intraperitoneally administrated naked CpG DNA cannot reach lymphatic organs. In fact, Agrawal et al. have reported that

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intraperitoneally administered 20 mer naked oligonucleotide distributed into the blood and distributed in the kidney as well as intravenously administered oligonucleotide [5]. Since liposome-entrapped compounds are selectively distributed to the lymphatic organs following intraperitoneal administration [6,7], CpG DNA/cationic liposomes complex (lipoplex) formation is expected to enhance the distribution of CpG DNA to lymphatic organs. Recently, we have reported that intraperitoneal administration of phosphodiester (PO) CpG DNA-lipoplexes ranging in size from 100 to 200 nm could efficiently inhibit peritoneal dissemination in a mouse model [8].

In this study, we found that phosphorothioate (PS)-CpG DNA-lipoplex produced better therapeutic effects on peritoneal dissemination than PO-CpG DNA-lipoplex in mice. As far as the mechanism of the antitumor activity of the PS-CpG DNA-lipoplex was concerned, we also found that PS-CpG DNA-lipoplex resulted in significantly higher cytokine production than PO-CpG DNA-lipoplex after being taken up by macrophages. PS-CpG DNA-lipoplex or PO-CpG DNA-lipoplex were administered intraperitoneally using a peritoneal dissemination model in mice which involved the intraperitoneal inoculation of colon26/Luc cells, a mouse colorectal adenocarcinoma cell line that stably expresses firefly luciferase gene. We previously reported that some tumor cells are present in the lymphatic system (greater omentum) 24 h after intraperitoneal inoculation of tumor cells [9]. Therefore, in order to ensure the adhesion and invasion of the tumor cells in the lymphatic organs, PS-CpG DNA-lipoplex was administered 3 days after tumor cell inoculation. The numbers of tumor cells were quantitatively evaluated by measuring the luciferase activity in the greater omentum and the mesentery as the index of peritoneal dissemination [10,11]. In addition, TNF-α contributes to the antitumor activity and, therefore, the TNF-α produced by immunocompetent cells in response to TLR-9 activation was measured using the primary cultured mouse peritoneal macrophages.

2. Materials and methods

2.1. Animals

Male Balb/c (6-week-old) mice and female ICR (4-week-old) mice were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Animals were maintained under conventional housing conditions. All animal experiments were approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences, Kyoto University.

2.2. Chemicals

RPMI1640 medium, phosphate buffered saline (PBS), Hanks' balanced salt solution (HBSS), and TGC medium were obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Opti-MEM I was obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was obtained from MP Biomedicals, Inc. (Irvine, CA). *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) was purchased from Tokyo Chemical Industry, Co., Ltd. (Tokyo, Japan) and cholesterol was from Nacalai Tesque Inc. (Kyoto, Japan). Oligonucleotides with phosphorothioate and phosphodiester backbones were purchased from Operon (Tokyo, Japan). The sequences of the oligonucleotides were 5′-TCGACGTTTTG-ACGTTTTGACGTTTT-3′ (CpG DNA) and 5′-TGCAGCTT-TTGAGCTTTTGAGCTTTT-3′ (control GpC DNA). The level of TNF-α from the culture supernatant was determined by the ELISA Ready-SET-go! set (eBioscience, San Diego, CA). All other chemicals were of the highest grade available.

2.3. Preparation of liposomes and their complex with CpG DNA

Cationic liposomes were prepared as reported previously [12]. In brief, DOTMA and cholesterol were mixed in chloroform at a molar ratio of 1:1, then the mixture was dried, vacuum-desiccated, and resuspended in 5% dextrose solution in sterile test-tubes. After hydration for 30 min at room temperature, the dispersion was sonicated for 10 min in a bath sonicator, then for 3 min in a tip sonicator to form liposomes and, finally, sterilized by passing through a 0.45-μm filter (Nihon-Millipore Ltd., Tokyo, Japan). Liposome/CpG DNA complexes were prepared as described in previous reports [8]. An equal volume of stock liposome solution and CpG DNA in 5% dextrose were mixed at various charge ratios, and left at 37 °C for 30 min. The mean particle size and ζ-potential of the lipoplexes were measured using a Zetasizer nano ZS instrument (Malvern Instruments, UK).

2.4. Cell lines

Murine adenocarcinoma colon 26 tumor cells [13] was grown in 5% CO_2 in humidified air at 37 °C with RPMI1640 medium supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Colon26 cells that stably express the firefly luciferase gene (colon26/Luc) were established as previously reported [14,15].

2.5. Peritoneal dissemination model and the antitumor activity of the CpG DNA-lipoplex

Colon26/Luc cells were trypsinized, and the cell concentration was adjusted to 10^6 cells/ml in HBSS. Then, 0.1 ml of the cell suspension was inoculated intraperitoneally into male Balb/c mice. Three days after tumor inoculation, 5% dextrose, naked PS- and PO-CpG DNA (1 μg/mouse), PS- and PO-CpG DNA-lipoplex (1 μg/mouse at the charge ratio 3.1) were administered to the peritoneal cavity of mice (0.2 ml 5% dextrose solution/mouse). Ten days after tumor inoculation, the mice were euthanized by cervical dislocation and the greater omentum and mesentery were excised and washed with ice-cold saline. Then, the organs were homogenized in a lysis buffer (0.05% Triton X-100, 2 mmol/l EDTA, 0.1 mol/l Tris pH 7.8), and centrifuged at 10,000 g for 10 min. Ten microliter of the supernatant was mixed with 100 μl luciferase assay buffer (Picagene, Toyo Ink,