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Poly (γ-glutamic acid) based combination of water-insoluble paclitaxel and TLR7 agonist for chemo-immunotherapy

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ABSTRACT

Advanced anti-cancer regimens are being introduced for more effective cancer treatment with improved life expectancy. In this research, immuno-stimulating agent toll-like receptor-7 (TLR-7) agonist-imiquimod and low dose chemotherapeutic agent-paclitaxel were synergized to demonstrate tumor therapy along with anti-tumor memory effect. Both therapeutic agents being water insoluble were dispersed in water with the help of water soluble polymer: poly (γ-glutamic acid) (γ-PGA) using a co-solvent systems leading to formation of micro-dispersions of drugs. Paclitaxel and imiquimod formed crystalline microstructures in the size range of 2–3 μm and were stably dispersed in γ-PGA matrix for more than 6 months. Paclitaxel and combination of paclitaxel and imiquimod had significant tumor killing effect *in-vitro* on various tumor cell lines, while antigen presenting cells (dendritic cells-DCs) treated with the same concentration of imiquimod along with the combination led to enhanced proliferation (250%). In DCs, enhanced secretion of pro-inflammatory and Th1 cytokines was observed in cells co-treated with paclitaxel and imiquimod dispersed in γ-PGA. When administered by intra-tumoral injection in mouse melanoma tumor model, the treatment with combination exemplified drastic inhibition of tumor growth leading to 70% survival as compared to individual components with 0% survival at day 41. The anti-tumor response generated was also found to have systemic memory response since the vaccinated mice significantly deferred secondary tumor development at distant site 6 weeks after treatment. The relative number and activation status of DCs *in-vivo* was found to be dramatically increased in case of mice treated with combination. The dramatic inhibition of tumor treated with combination is expected to be mediated by both chemotherapeutic killing of tumor cells followed by uptake of released antigen by the DCs and due to enhanced proliferation and activation of the DCs.

1. Introduction

Cancer continues to be one of the leading cause of death, accounting for more than 1.5 million new cases and 0.5 million deaths [1]. Tumor is a complex and heterogeneous structure involving co-evolution of vasculature, tumor-resistant immune cells, extracellular matrix including fibroblasts which assist tumor cells escape therapeutic intervention [2,3]. While chemotherapy has been used as a prominent anti-cancer modality, anti-cancer drugs are known to have high incidence of side-effects and disease recurrence [4]. Paclitaxel is a potent chemotherapeutic agent used for treatment of various cancers such as breast, non-small cell lung cancers, ovarian cancer, malignant brain tumors, and a variety of other solid tumors [5]. The clinical efficacy is jeopardized because of the systemic side-effects, insolubility in water and low bioavailability of paclitaxel [6,7]. While various new approaches for effective and targeted delivery of paclitaxel are in progress [8,9], it has become a desirable candidate drug for combination with various other modalities, but has gained limited success in clinical trials [10–13].

Immune system is indispensable aspect of tumor microenvironment because tumor has the ability to evade the immune system by various mechanisms such as suppression of tumor reactive T cells by transforming growth factor-β (TGF-β) secretion or through regulatory T cells or by direct upregulation of death ligands such as Fas-L [14]. Consequently, immunotherapeutic approaches for cancer such as dendritic cells and T cells based vaccine, cytokines, toll-like receptor (TLR) agonists, viral vaccine, peptide based vaccine, DNA based vaccines have gained importance [15,16]. Lately, the role of TLR stimulation has been emphasized for cancer

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treatment [17–20] and combination of TLR agonists with other treatment modalities such as T cell modulation, anti-CTLA4 therapy or CD40L plasmid DNA for cancer treatment has been reported [21–23]. While TLR agonists are being explored for anti-cancer treatment, on a contrary, cancer cells are known to express TLRs and TLR agonists are also found to facilitate tumor proliferation, metastasis and inhibit apoptosis [24]. Thus, selection of suitable TLR agonist for cancer therapy is a critical step. TLR-7 agonist imiquimod also known as R837 has been approved by FDA for topical administration in cancer therapy and diseases such as genital warts, actinic keratoses, superficial basal cell carcinoma and lentigo maligna [25,26]. Imiquimod is capable of imparting cytotoxic T cells with enhanced anti-tumor properties [27]. Imiquimod is also known to induce systemic immunity in cryosurgery patients [28]. In clinical trials, although it was well-tolerated with minimum side-effects, effective therapeutic response was not observed [29]. Because of the dual nature of TLR agonists in cancer, monotherapy using standalone TLR agonists is leading to inadequate treatment and has great scope for improvisation [30,31].

Taking into consideration the limitations of chemotherapy and immunotherapy, chemo-immunotherapy has emerged as a new branch of cancer research with highly promising results [32]. The efficacy of chemotherapeutic agent can be increased if the host immunity is also taken into consideration. It has been found in phase III trial using combination of chemotherapeutic agent 5-fluorouracil (5-FU) and adriamycin with TLR3 agonist polyadenylic-polyuridylic acid (poly A:U) leads to significantly prolonged patient survival as compared to chemotherapy alone [33]. In a study, combination of paclitaxel and TLR4 agonist illustrated 40% reduction in tumor in mice as compared to paclitaxel alone [34]. In another report, it was demonstrated that pre-conditioning with chemotherapeutic agent complemented by adoptive T cell transfer, viral vaccine and immunostimulatory TLR agonist is capable of getting rid of melanoma tumor completely [35]. These results suggest that if the tumor surveillance is broken, then even low concentrations of anti-cancer drug is expected to be effective with reduced side-effects. However, the challenge is to determine an appropriate combination of anti-cancer drug and TLR agonist, which can completely eliminate the tumor without any reappearance of the disease. Another consideration is that anti-cancer drug should have minimum adverse effect on the immune cells while it should be able to kill tumor cells at the same concentrations of treatment. Also, the TLR agonist selected should not only be able to counter the immuno-suppressive environment within the tumor but should also be capable of triggering release of cytokines by immune cells which is sufficient for development of anti-tumor milieu.

In this research, poly (γ-glutamic acid) (γ-PGA) based combination of low dose of anti-cancer drug (paclitaxel) with immune-stimulatory agent (imiquimod) was tested for synergetic effect against solid tumor. Paclitaxel treatment was speculated to cause tumor cell death which should lead to production of tumor specific antigens as well as danger signals also known as damage associated molecular patterns (DAMPs). Imiquimod was used as an adjuvant and was expected to induce activation and maturation of immune cells for eliciting anti-tumor immune response. Since, both paclitaxel and imiquimod are water-insoluble drugs, γ-PGA which is a water soluble bioderived anionic polymer was used to form a stable aqueous micro-dispersion of the two drugs. The memory response against tumor was also assessed in order to test the longevity and clinical applicability of the formulation.

2. Materials and methods

2.1. Preparation of polymer-drug micro-dispersion

For preparation of γ-PGA-paclitaxel micro-dispersion (γ-PGA/Ptx), 10 mg of paclitaxel (ChemieTek, Indianapolis, USA) in 500 μl DMSO was added to 2 ml of 2.5% (w/v) of 50 kDa poly (γ-glutamic acid) (BioLeaders Corporation, Daejeon, South Korea) while sonicating using a probe sonicator for 2 min. For preparation of γ-PGA-imiquimod micro-dispersion (γ-PGA/Imq), 10 mg of imiquimod (TCI, Tokyo, Japan) was dissolved in 2 ml DMSO by heating at 60 °C. This solution was added to 2 ml of 2.5% of 50 kDa γ-PGA while sonicating using a probe sonicator for 2 min. The above solutions were lyophilized for removal of organic solvents. The lyophilized powder was redispersed in distilled water to get a stable dispersion. For preparation of γ-PGA-paclitaxel-imiquimod micro-dispersion (γ-PGA/Ptx/Imq) for combination therapy the redispersed solutions made above were mixed in ratio 1:5 (γ-PGA/Ptx: γ-PGA/Imq).

2.2. Characterization of micro-dispersion

The size of the redispersed solutions was measured using Electrophoretic Light Scattering-ELS Z (Otsuka Electronics, Osaka, Japan). The stability of the dispersion was assessed by observing the samples for aggregation and phase separation after storing the sample at 4 °C for 1 week after dispersion in distilled water. The morphology of theses micro-dispersions were observed through FE-SEM (JSM-7000F, JEOL Ltd. Japan) and FE-TEM (JEM-2100F HR, JEOL Ltd. Japan). For the SEM sample preparation, lyophilized and redispersed suspension was placed on a silicon-wafer plates and vacuum dried to remove water, followed by platinum coating using a Technics Hummer II sputter coater for 90 s at 30 mA. For TEM sample preparation a drop (2 μl) of redispersed suspension was placed onto a formvar/carbon coated copper grid, followed by vacuum drying to evaporate water.

2.3. In-vitro viability assay

Tumor cells (A549 human lung adenocarcinoma epithelial cell line, B-16 murine melanoma cell line, HeLa human cervical cancer cell line) and immune cells (bone marrow derived dendritic cells-BMDCs and RAW264.7 macrophages) were tested for *in-vitro* viability after treatment with various concentrations of γ-PGA/Ptx, γ-PGA/Imq and γ-PGA/Ptx/Imq. All tumor cell lines and RAW264.7 were purchased from ATCC. BMDCs were isolated from mouse bone marrow as reported previously [36]. MTS assay for analysis of mitochondrial activity was used as a measure of cell viability. 1×10^4 cells in 100 μl media were seeded per well in a 96 well plate (Corning Costar, Cambridge, MA, USA). 100 μl of sample dispersed in cell culture media was added and incubated for 24 and 48 h at 37 °C and 5% CO_2. After incubation, 20 μl of MTS solution-Cell Titer 96 Aqueous One Solution kit (Promega, Madison, WI, USA) was added to each well and incubated for 2 h. The absorbance of the samples was taken at 490 nm (VersaMax, Molecular Devices, Sunnyvale, CA, USA) and was normalized with respect to absorbance of untreated cells.

2.4. In-vitro BMDC activation and maturation

1×10^6 BMDCs in 1 ml of RPMI media supplemented with 10% FBS and 1% antibiotic-antimycotic solution were seeded per well in a 6 well plate (Corning Costar, Cambridge, MA, USA). 1 ml of sample was treated and cells were incubated for 24 h at 37 °C and 5% CO_2. The plates were centrifuged at 1500 rpm for 5 min and the supernatant was collected and was analyzed for various cytokines (IL-1β, IL-12, IL-6 and TNFα) using ELISA kit (BD Biosciences). For analysis of upregulation of maturation marker after incubating with samples for 24 h, the cells were harvested and fixed using 4% paraformaldehyde. They were treated with fluorescence labeled marker antibody FITC-CD80 (BD PharMingen, San Diego, CA, USA), PE-MHC I (eBioscience, San Diego, CA, USA) and PE-MHC II (eBioscience, San Diego, CA, USA) by incubating at 4 °C for 30 min followed by 2 times washing with PBS. 10^4 cells were then analyzed for fluorescence using MACS (Miltenyl Biotec). Data was further analyzed using MACSQuant Analyzer (Milteny Biotec, Germany).

2.5. Mice and cell lines

5 to 6 weeks old female C57BL/6 (H-2b) mice were purchased from KOATECH (Pyeongtaek, Korea). The mice were maintained under pathogen-free condition. All experiments employing mice were performed in accordance with the Korean NIH guidelines for care and use of laboratory animals. B16–F10 which is a murine melanoma cell line was purchased from ATCC. It was allowed to grow in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic solution at 37 °C with 5% CO_2/95% humidified air.

2.6. Evaluation of in-vivo antitumor activity

B16-F10 melanoma cells (1×10^5) were inoculated into the right flank of 5–6 weeks old C57BL/6 mice. After 1 week of tumor implantation, animals with an average tumor diameter of 4–6 mm were selected. These mice were divided into five treatment groups ($n = 7$) and were numbered. Each drug was administered by intra-tumoral injection. The drug treatment was continued from day 7 after tumor implantation to day 19 with 3 day interval. Total 4 injections were given (day 7, 11, 15 and 19). The tumor diameters were measured till 23rd day after tumor implantation using a sliding caliper. Tumor volume was calculated using the following formula: tumor volume (mm^3) = length × $(\text{width})^2/2$. On day 23 mice were euthanized and the tumors were dissected and photographed. For survival study, the treated animals were observed for 41 days. For secondary tumor challenge, tumor-free mice at