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Neem leaf glycoprotein is superior than Cisplatin and Sunitinib malate in restricting melanoma growth by normalization of tumor microenvironment

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ABSTRACT

We have observed earlier that therapeutic treatment with neem leaf glycoprotein (NLGP) inhibits murine B16-melanoma growth *in vivo* and improves survivability of treated mice. Anti-tumor effect of NLGP is directly associated with enhanced $CD8^+$ T cell activity and downregulation of suppressive cellular functions. Objective of this present study is to know the efficacy of NLGP in comparison to two popular drugs, Cisplatin and Sunitinib malate (Sutent) in relation to the modulation of tumor microenvironment (TME). Analysis of cytokine milieu within TME revealed IL-10, TGFβ, IL-6 rich type 2 characters was significantly switched to type 1 microenvironment with dominance of IFNγ and IL-2 within NLGP-TME, which was not found in other cases; however Cisplatin-TME appeared better in type 2 to type 1 conversion than Sutent-TME as evidenced by RT-PCR, ELISA and immunohistochemical analysis. NLGP-TME educated $CD8^+$ T cells exhibited greater cytotoxicity to B16 Melanoma cells *in vitro* and these cells showed comparatively higher expression of cytotoxicity related molecules, perforin and granzyme B than Cisplatin-TME and Sutent-TME educated T cells. Adoptive transfer of NLGP-TME exposed T cells, but not PBS-TME exposed cells in mice, is able to significantly inhibit the growth of melanoma *in vivo*. Such tumor growth inhibition was in significantly lower extent when therapeutic $CD8^+$ T cells were exposed to either Cisplatin-TME or Sutent-TME or control-TME. Accumulated evidences strongly suggest that non toxic NLGP normalized TME allows T cells to perform optimally than other TMEs under study to inhibit the melanoma growth.

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1. Introduction

Cisplatin, a platinum containing anti-cancer drug, has long been used for treatment of various types of human malignancies [1]. It acts by inhibiting the proliferation of tumor cells by attenuating DNA binding preferentially with guanine base. This causes formation of $[PtCl(guanine\text{-}DNA)(NH_3)_2]^+$, which crosslinks via displacement of the other chloride ligands, typically by another guanine that in turn activate apoptosis [2]. Cell proliferation is not influenced at mitosis but during DNA replication, which is initially blocked by inter and intra-DNA crosslinks. Sunitinib malate (Sutent), is a tyrosine kinase inhibitor, presently in clinic for the treatment of renal cell carcinoma [3] and gastrointestinal stromal tumor [4]. It acts by inhibiting regulatory cells signaling, targeting multiple receptor tyrosine kinases (RTKs) along with c-kit/CD117 [5]. Besides established anticancer therapeutics, Cisplatin and Sutent, we have recently reported that NLGP can restrict the murine tumor growth in therapeutic settings, without direct tumor cell killing by NLGP, but through activation of $CD8^+$ T cell mediated antitumor immunity, particularly within tumor microenvironment (TME).

Tumor cells are lodged in TME having several tumor promoting cellular and soluble factors. Targeting of tumor cells by any drug may kill them, but promotes several protumor functions [6]. The microenvironment of tumor, which differ significantly from normal tissue microenvironment also severely impairs the treatment efficacy of conventional anticancer therapies. Thus, targeting the TME becomes an increasingly logical and attractive therapeutic option in cancer management. In our recent studies, NLGP mediated normalization of TME is observed that includes elevated functions of $CD8^+$ effector T cells, type I cytokine dominance and subdued activity of suppressor cells within TME. Sutent has some role in normalization of TME by targeting tumor-associated enhanced VEGFR/PDGFR signaling and also by decreasing number of Treg and MDSC within TME [7] and detailed study is still awaiting. On the other hand, in spite of tumor cytolytic

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action of Cisplatin, little is known regarding effect of Cisplatin on TME. Its prominent effect on induction of leukepenia indirectly suggests the creation of pro-tumor microenvironment [8] following Cisplatin chemotherapy.

In this perspective, present comparative study is aimed to know the mechanism of action of three tumor restricting molecules in view of the normalization of TME, especially in terms of cytokine profiling and ability to activate $CD8^+$ T cells both *in vitro* and *in vivo*. Systemic toxicity of these three anti-tumor molecules is also evaluated to ascertain their scope as anti-tumor agent in future medicine.

2. Materials and methods

2.1. Reagents and antibodies

Roswell Park Memorial Institute (RPMI) 1640 and Dulbecco's Modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies (NY, USA). Lymphocyte separation media (LSM) was obtained from MP Biomedicals (Solon, OH, USA). $CD8^+$ T cell isolation kit was procured from Miltenyi Biotec (GmbH, Germany). IFNγ/IL-10 estimation kits, 3, 3´, 5, 5´-tetramethylbenzidine substrate solutions were obtained from BD Pharmingen, USA. Cytotoxicity detection kit based on lactate dehydrogenase (LDH) release was procured from Roche Diagnostics (Mannham, Germany). Western lighting chemiluminescence detection kit was purchased from Pierce (Rockford, IL, USA). Purified monoclonal antimouse IL-10, IL-12 and TGFβ were procured from e-Biosciences. Reverse transcription-PCR (RT-PCR) primers were designed and procured from MWG-Biotech AG (Bangalore, India). Cisplatin (cis-Diamineplatinum (II) dichloride) was purchased from Sigma-Aldrich, USA and Sunitinib malate (Sutent™) was obtained from Pfizer, New York, NY.

2.2. Neem leaf glycoprotein (NLGP)

Extract from neem (*Azadirachta indica*) leaves was prepared by the method as described earlier [9,10]. Mature leaves of same size and color (indicative of same age), taken from a standard source were shed-dried and pulverized. Leaf powder was soaked overnight in phosphate buffered saline (PBS); pH 7.4, supernatant was collected by centrifugation at 1500 rpm. Neem leaf preparation (NLP) was then extensively dialyzed against PBS, pH 7.4 and concentrated by Centricon Membrane Filter (Millipore Corporation, Bedford, MA, USA) with 10 kDa molecular weight cut off. Endotoxin content of the freshly prepared NLP was determined by Limulus Amebocyte Lysate (LAL) test as per manufacturer's (Salesworth India, Bangalore) instruction. The endotoxin content of all the batches of NLP (extract obtained from 0.25 mg leaf powder in 1 ml PBS) was found to be less than 6 pg/ml. Glycoprotein present in this preparation (Neem leaf glycoprotein-NLGP) was isolated and characterized by the method described [10,11]. The purity of NLGP was checked by Size Exclusion-HPLC (SE-HPLC) in a protein PAK 300 SW column of 7.5 mm (ID) × 30 cm. The glycoprotein was eluted using gradients of PBS at a constant flow rate of 1.0 ml/min under a pressure of 3×10^6 N/m^2. We used 20 μg of BSA (Sigma, USA) to standardize the retention time of the protein within the column. The protein peaks were determined by absorption at 280 nm in a UV recorder [12].

2.3. Animals and tumor cells

Female C57BL/6 mice having average age of 6 weeks, body weight 25 g were obtained from Institutional Animal Facilities, CNCI, Kolkata, India. Autoclaved dry pellet diet (Epic Laboratory Animal Feed, Kalyani, India) and water were given *ad libitum*. Maintenance and treatment of animals were given according to the guidelines established by the Institutional Animal Care and Ethics Committee. Solid melanoma tumors were developed in C57BL/6 mice by inoculation of 2×10^5 B16 melanoma F10 cells subcutaneously into syngenic mice and allowed to grow as solid tumor. Tumors were removed from diseased mice and used in different experiments as described below.

2.4. Tumor cell lines

The B16 melanoma F10 cell line was maintained in DMEM high glucose supplemented with 10% heat inactivated FBS, 2 mM L-Glutamine, penicillin (50 units/ml) and streptomycin (50 μg/ml) at 37 °C with the supply of 5% CO_2.

2.5. Drugs and treatments

Mice having tumor of average size 260 mm^3 were treated with different drugs. NLGP was injected subcutaneously into tumor bearing mice (25 μg/mice/injection) once in a week for four weeks in total. Cisplatin was injected intra-peritoneal in a dose of 0.1 mg/mice/injection. Total five doses were given in one day interval. Sutent was injected orally at a dose of 0.5 mg/mice/injection for consecutive 7 days. Tumors were harvested from mice with different treatment at day 20 post tumor inoculation.

2.6. Hematological assessment

Blood was collected weekly by retro-orbital puncture after ether anesthesia from mice and used for hematological assessment. Total Red Blood Cells (RBC), White Blood Cells (WBC), platelet count and hemoglobin content were assessed in an autoanalyzer (Sysmex, Japan). Neutrophils and lymphocytes were counted from the blood smear after Leishman's staining.

2.7. Tumor microenvironment

Tumor tissues were harvested from both NLGP and Phosphate Buffered Saline (PBS) treated mice and weighed. The identical weight of tumor tissues from each group of mice was minced using sterile scalpel and exposed to repetitive freeze-thaw cycles as described [13,14]. Prepared lysates were centrifuged at 10,000 rpm for 10 min and supernatant was collected to use as tumor microenvironment (TME). TME from either PBS or NLGP treated mice was designated as PBS-TME and NLGP-TME respectively. These TME were used for *in vitro* treatment and estimation of soluble cytokines and growth factors. For western blot analysis after repetitive freeze-thaw cycles tumors were dissolved in Radio Immuno-Precipitation Assay buffer (RIPA buffer), kept at 4 °C for 30 min and after centrifugation supernatants were collected. In every case protein concentration of the preparation was measured by using Folin-phenol reagent [15].

2.8. Cytokine detection assay

To quantify cytokines, solid tumors harvested at different days after tumor inoculation and TME was prepared. Secretion of different cytokines (IFNγ, IL-12p40, IL-2, IL-10, IL-6, TGFβ and VEGF) within TME was assessed by ELISA and optical density was measured at 450 nm using microplate reader (BioTek Instruments Inc., Vermont, USA).

2.9. TME educated effector cells

Spleens were isolated from normal mice. Splenic mononuclear cells (1X10^6 cells) were purified by density gradient centrifugation on LSM and exposed to either PBS-TME or NLGP-TME (10 μg of protein) in complete RPMI 1640 media for 120 h at 37^{0}C with supply of 5% CO_2. After incubation, non-adherent fractions were collected as effector cells. $CD8^+$ T cells were purified from effector cells using magnetic activated cell sorter (MACS), (Milltani Biotech Inc., CA, USA), by the method described below.

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