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Noble metal nanoparticle-induced oxidative stress modulates tumor associated macrophages (TAMs) from an M2 to M1 phenotype: An *in vitro* approach

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ABSTRACT

Diagnosis of cancer and photothermal therapy using optoelectronic properties of noble metal nanoparticles (NPs) has established a new therapeutic approach for treating cancer. Here we address the intrinsic properties of noble metal NPs (gold and silver) as well as the mechanism of their potential antitumor activity. For this, the study addresses the functional characterization of tumor associated macrophages (TAMs) isolated from murine fibrosarcoma induced by a chemical carcinogen, 3-methylcholanthrene (MCA). We have previously shown antitumor activity of both gold nanoparticles (AuNPs) and silver nanoparticle (AgNPs) in vivo in a murine fibrosarcoma model. In the present study, it has been seen that AuNPs and AgNPs modulate the reactive oxygen species (ROS) and reactive nitrogen species (RNS) production, suppressing the antioxidant system of cells (TAMs). Moreover, the antioxidant-mimetic action of these NPs maintain the ROS and RNS levels in TAMs which act as second messengers to activate the proinflammatory signaling cascades. Thus, while there is a downregulation of tumor necrosis factor-α (TNF-α) and Interleukin-10 (IL-10) in the TAMs, the proinflammatory cytokine Interleukin-12 (IL-12) is upregulated resulting in a polarization of TAMs from M2 (*anti*-inflammatory) to M1 (pro-inflammatory) nature.

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1. Introduction

Over a century the most controversial issue in immunology has been the possibility of mounting an effective immune response against malignant tumor. There have been several theories associated with immunotherapy and cancer [1]. Of the most promising theories, a potent option is that recruitment of tumoricidal myeloid cells into tumors leads to inflammatory mechanisms that recruit hypoxia-induced ligands on tumor cells and generate antiangiogenic immune responses [1,2]. The formation of malignant tumor at the site of chronic inflammation establishes the cardinal link between inflammation and cancer. The relationship between inflammation, cancer and innate immunity is widely accepted due to production of a number of chemokines and cytokines by tumor cells that attract leukocytes to the inflamed area [3]. Inflammatory components of a developing tumor may include diverse population of dendritic cells, neutrophils, eosinophils, macrophages, mast cells as well as lymphocytes, all of which are capable of producing an assorted array of cytokines and cytotoxic mediators including reactive oxygen species (ROS), reactive nitrogen species (RNS), cysteine and serine proteases. One of these, the macrophage, has emerged as the central regulatory cell populace for tumor induction and promotion [4,5]. Macrophages appear to contribute to neoplastic transformations at the site of chronic inflammation, where these macrophages establish tumor sustenance by inducing tumor growth, angiogenesis and metastasis [6,7]. Since these macrophages remain associated with the tumor, they have been coined as tumor associated macrophages (TAMs). TAMs exhibit the M2 phenotype characterized by the anti-inflammatory molecules like Interleukin-4 (IL-4) as well as the proinflammatory Interleukin-6 (IL-6), TNF-α and Interleukin-10 (IL-10) and are characterized by their scavenging potential, ability to support angiogenesis, tissue remodeling, enhanced release of anti-inflammatory cytokines and immunosuppressive action [7]. In contrast, the M1 phenotype is characterized by the release of regulatory cytokines, ROS, RNS and tumoricidal/microbicidal activity as existing in non-tumorous systemic macrophages [8,9].

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There have been several approaches including chemotherapy and gene therapy (microRNA) to ameliorate tumors by attempting to repolarize TAMs from M2 phenotype to M1 phenotype, which were typically limited by their toxicity, short circulation half-life in plasma, and non-selectivity [2,10,11]. Immunologists and oncologists have been searching for an alternative answer for long when nanotechnology gave them the opportunity to diagnose and treat cancer by applying new imaging agents and multifunctional, targeted devices capable of crossing biological barriers to deliver therapeutic agents directly to cells and tissues involved in cancer growth and metastasis. As a result, there has been a redeeming hope that nanotechnology could become a reality in cancer immunotherapy. Additionally, the nanoparticle (NP)-based systems can provide with simultaneous diagnosis and therapy, i.e., theranostics, exploring their unique properties like high surface to volume ratio, facile surface chemistry and functionalization for better penetration of drugs and their tracking within the body, which allow for a more efficient therapeutic measure with comparatively reduced risks than conventional therapies [12]. Noble metal NPs such as gold and silver are particularly interesting due to their size and shape dependent unique optoelectronic properties [13]. The optoelectronic properties of such noble metal NPs open the door for cancer theranostics. However, the intrinsic properties of noble metal NPs in the tumor microenvironment are yet to be fully elucidated. There are a handful of reports available on toxicity, which depend on shape, size, time and route of exposure of NPs [14] as well as their biocompatibility, where it has been shown that gold nanoparticles (AuNPs) are nontoxic, non-immunogenic and highly permeable to cells without damaging their functionality [15]. In an experiment with an ear model, it was shown that AuNPs as well as silver nanoparticles (AgNPs) have *anti*-angiogenic property [16,17]. In a previous study, we have investigated the potential intrinsic antitumor property of mouse serum albumin (MSA) coated AuNPs (AuNP-MSA) and AgNPs (AgNP-MSA) highlighting their role in eliciting oxidative stress and immunological interference in a tumor fibrosarcoma model [18]. Here, we address the detailed mechanism of functional polarization from M2 (anti-inflammatory/protumorogenic) to M1 (pro-inflammatory/antitumorogenic) phenotype of TAMs by AuNP-MSA and AgNP-MSA.

2. Methods and methodology

2.1. Synthesis of MSA-stabilized AuNPs

A wide range (size) of monodispersed gold colloids can be synthesized by varying [Au (III)]/[citrate] ratio during the reduction step by Frens method, employing trisodium citrate as the surface capping as well as a reducing agent [19,20]. In this procedure, 10 nm particles have been synthesized by adding 1.6 ml trisodium citrate solution (1%) to the 50 ml Gold (III) chloride ($HAuCl_4$) solution (0.25 mM) under boiling condition. The pale yellow solution turned to faintly blue color within 25 s indicating the nucleation, which rapidly changed to brilliant red coloration at about 70 s displaying the formation of the particles. The particles were allowed to stand for 24 h before use. Then the particles were functionalized by 'ligand exchange method' [21] with MSA in a typical reaction; 25 mL (5 mM) MSA solution was added to 25 ml citrate stabilized AuNPs and stirred overnight at room temperature. The MSA stabilized AuNPs were washed with Milli-Q water by centrifugation and re-dispersed in water to remove the trisodium citrate and excess MSA present in the solution.

2.2. Synthesis of AgNPs stabilized with MSA via ligand exchange method

AgNPs have been synthesized under controlled condition by sodium borohydride ($NaBH_4$) reduction. In this method, 250 μl $NaBH_4$ solution (0.1 M) was added to 50 ml silver nitrate solution (0.5 mM) under vigorous stirring condition in well a capped round bottom flask for 30 min. The colorless solution turned to straw coloration indicating the formation of AgNPs. Next, ligand exchange methods [21] employed for the synthesis of MSA stabilized AgNPs. In this method, 1 ml 0.5 M MSA solution was added to AgNPs solution and stirred vigorously in open condition at room temperature (298 ± 1 K) for 3 h. During this process, the replaced borate ions decomposed to diboron tetrahydride (B_2H_4) gas and were removed from the solution. The AgNP-MSA were washed with Milli-Q water by centrifugation and re-dispersed in water to remove the excess MSA present in the solution.

2.2.1. Characterization of AuNP-MSA and AgNP-MSA

Characterization of AuNP-MSA and AgNP-MSA were carried out using UV Spectra, and Fourier Transformed Infrared Spectroscopy (FT-IR) analysis. Loading of MSA on the NPs was appraised from the entrapment efficiency (EE) estimated spectrophotometrically at 595 nm using the following equation [22]

$$EE = (\text{Total initial amount of MSA added} - \text{Free amount of MSA in supernatant}) \times (\text{Total initial amount of MSA added})^{-1} \times 100$$

After that, size and shape on NPs were analyzed by transmission electron microscopy (JOL, JSM-2100, JEOL, Japan) at the Sophisticated Analytical Instruments Facility (SAIF), North East Hill University (NEHU), Shillong, Meghalaya, India.

2.3. Animals

6 to 7 week old LACA male Swiss albino mice having body weight of 20 ± 2 g were purchased from Pasteur Institute, Shillong, India (License no.: 34/DR/1966).The mice were accommodated in polycarbonate cages at 22 ± 2^{0}C temperature, 85% relative humidity and 12 h light-dark cycles with standard food and water ad libitum. All treatments were as per the guidelines of the Institutional Ethical Committee (IEC/AUS/2013–019 dated: 20–03–2013).

2.4. Isolation of splenic macrophages

After sacrifice of experimental mice, spleens from all the mice were excised and placed in Alsever's solution. The single cell suspension of spleens were prepared by maceration followed by isolation of macrophage were carried out according to the method of Chakraborty and Sengupta (2012). Finally macrophages were collected and transferred for viability check by Trypan blue exclusion technique [23].

2.5. Isolation of TAMs

To develop the fibrosarcoma model, mice were injected with a single dose (0.5 mg/mice) 3-methylcholanthrene (MCA) subcutaneously at the right flank and 4.26 nM phorbol myristate-13-acetate (PMA) at the opposite flank for 40 weeks on alternate days [18]. After 40 weeks, mice were sacrificed and solid tumors were taken out from the flanks. The tumors were cut into 1mm^3 fragments followed by collagenase [0.3% collagenase in Dulbecco's phosphate-buffered saline (DPBS)] digestion for 45 min at 37 °C. To get a single cell preparation, the cell suspension digested with collagenase was filtered through 70 μm stainless steel wire mesh. The single cell suspension was twice washed with DPBS. Then the cells were resuspended in serum-free Roswell Park Memorial Institute 1640 (RPMI 1640) medium and left to adhere in plastic petri dishes at 37 °C in 5% CO_2 for 1 h. The non-adherent cells were washed away and the remaining 95% adherent cells were TAMs as considered by morphology and macrophage specific marker CD68 [24].

2.6. Selection of samples for in vitro experiment

Three groups of samples were taken from LACA Swiss albino mice (body weight 20 ± 2 g and 6–7 weeks old) from respectively normal and tumor-bearing genres.

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