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Assessing the immunomodulatory role of heteroglycan in a tumor spheroid and macrophage co-culture model system

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ABSTRACT

The therapeutic benefits of glycans have garnered much attention over the last few decades with most studies being reported in 2D cultures or in animal models. The present work is therefore aimed to assess the effects of an immunomodulatory heteroglycan in a 3D milieu. Briefly, HT29 tumor spheroids were incubated with THP-1 macrophages at 1:1 ratio in a culture medium supplemented with immune stimulants such as heteroglycans or LPS. Spheroidal distortion, migration of tumor cells from the periphery of the spheroids and 46% of higher macrophage invasiveness was noted in heteroglycan-treated co-cultures with respect to control cultures. Histological sections of the treated co-cultures revealed the presence of high apoptotic tumor cells in the spheroidal periphery. CD11c and CD68 staining further suggested the predominance of macrophages in the vicinity of the apoptotic tumor cells. Such an *in vitro* created tissue system may thereby confirm the effectiveness of heteroglycan in activating the immune cells to exhibit tumor cytotoxic properties.

1. Introduction

Most biological studies till date have focussed on two dimensional (2D) models to delineate cellular and molecular cues which occur in living cells in disease conditions or when subjected to therapeutics. Such 2D models often have limitations with respect to cell–cell and cell–matrix interactions and thereby do not epitomize the complex *in vivo* system. To account for the micro environmental influences of cells in a tissue system, a compact three dimensional (3D) spheroidal aggregation of cells that exhibit intermediate complexity may serve as an alternative to the *in vivo* models and thereby bypass the time taking animal-based experiments. These multicellular tumor spheroids (MCTS) may be considered to be biomimetic in nature and can unravel various characteristics such as growth kinetics, physiological milieu (nutrient supply, pH distribution, etc.) and the effects of drugs or other effector molecules in cancer cells (Audran, Dazord, & Toujas, 1994; Barbone, Yang, Morgan, Gaudino, & Broaddus, 2008; Gottfried, Kunz-Schughart, Andreesen, & Kreutz, 2006). Co-culturing of tumor spheroids with immune cells such as macrophages, NK cells or T-cells have also been in common application during 1990s and has currently re-entered the scene in the last few years to investigate new immunotherapies (Hirschhaeuser et al., 2010). Although tumor spheroid may not reflect the exact *in vivo* system, it still possesses tissue-like properties such as compactness, chemical gradients, growth kinetics as well as cell–matrix and cell–cell interactions (Hirschhaeuser et al., 2010). In such co-culture systems, the properties of activated immune cells may be monitored by studying parameters such as migration, invasiveness, anti-tumorigenicity and thereby serve as a strong ground for effectively screening the potency of therapeutic molecules (Audran et al., 1994; Konur, Kreutz, Knuchel, Krause, & Andreesen, 1996). Loss of spheroidal membrane integrity is also used as a classical endpoint to relate the impact of treated to untreated co-cultures (Friedrich, Seidel, Ebner, & Kunz-Schughart, 2009).

The role of lipopolysaccharides (LPS) in modulating the function of macrophages when co-cultured with MCTS of J82 urothelial carcinoma have revealed that LPS can induce macrophages to execute a cytotoxic effect on tumor cells (Konur et al., 1996; Konur, Kreutz, Knuchel, Krause, & Andreesen, 1998). Macrophages may be well defined as the 'big eaters' of the innate immune system. Circulating monocytes on differentiating into tissue macrophages gain the potency to engulf apoptotic cells and are poised to secrete effector molecules which cause macrophage mediated anti-tumorigenic effects. These macrophages are often plastic in nature and can modulate their biofunctional phenotype based on the environmental cues (Murray & Wynn, 2011). There also exist reports which suggest that tumor-associated macrophages (TAMs) may plausibly

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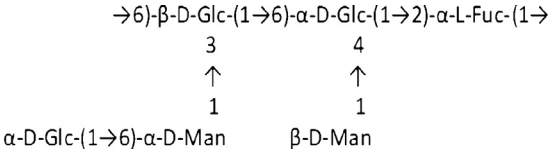
either function to be anti-neoplastic or support tumor progression depending on the presence or absence of certain stimuli (Mantovani, Bottazzi, Colotta, Sozzani, & Ruco, 1992). Furthermore, in some organotypic co-cultures it has been observed that the presence of IFN-γ or LPS in the culture medium led to M1 polarization of macrophages and concomitantly exhibited tumor inhibition (Linde, Gutschalk, Hoffmann, Yilmaz, & Mueller, 2012). Based on these reports, our present work is thereby focussed on evaluating the role of an immunomodulatory heteroglycan which can stimulate macrophages to cause immune cell mediated tumor cytotoxicity. Heteroglycans have recently been one of the most promising alternatives to classical chemotherapy as they direct their efficacy by augmenting host immune responses (Schepetkin & Quinn, 2006; Uthaisangsook, Day, Bahna, Good, & Haraguchi, 2002). These biomolecules exhibit a wide range of therapeutic properties with relatively low toxicity to normal cells (Wasser, 2002). Till date, all studies related to the efficacy of heteroglycans have been performed in the *in vitro* or mice models. An attempt to reproduce the biological function of heteroglycans in an *in vitro* three dimensional tumor spheroid and macrophage co-culture model will thereby provide a clear idea of the interactions that occur between activated macrophages and tumor tissues.

In the present work, the immune stimulating trait of a heteroglycan isolated from the mycelia of *Pleurotus ostreatus* (a commonly cultivated therapeutic mushroom) was investigated in a 3D tumor spheroid and macrophage co-culture milieu. The heteroglycan has been physiochemically characterized and its potency in stimulating the immune cells has already been investigated in RAW264.7 murine cell line as well as in tumor induced mice models (Devi et al., 2013). The isolated heteroglycan molecule had a basic composition of glucose, mannose and fucose in a 3:2:1 ratio with branching at (1 → 2), (1 → 3), (1 → 4) and (1 → 6) sites (Devi et al., 2013; Patra et al., 2013) and may be presented in a molecular fashion as follows:



Three dimensional studies were performed by incubating HT-29 tumor spheroids with THP-1 human macrophages at a 1:1 cell ratio in a medium supplemented with the isolated heteroglycan. Discrete factors which govern macrophage stimulation such as chemotaxis, migration, invasiveness and tumor cytotoxicity were noted to be enhanced in heteroglycan stimulated cultures when compared to control co-cultures with no heteroglycan supplementation thereby signifying the reproducibility of *in vitro* or *in vivo* effects of the heteroglycan in a confined three dimensional micromilieu.

2. Materials and methods

2.1. Chemicals, equipments and isolation of heteroglycan

Roswell Park Memorial Institute (RPMI) medium and Foetal Bovine Serum (FBS) for cell culture purposes were obtained from Gibco. Antibiotics, trypsin EDTA, glass slides, sodium pyruvate and beta-mercaptoethanol were purchased from Himedia. Cytotracker green and red were procured from Invitrogen while lipopolysaccharide (*Salmonella typhimurium* origin) and APTES (3-Aminopropyl triethoxysilane) were obtained from Sigma, USA. Other necessary items such as formaldehyde, xylene and haematoxylin were taken from Merck and eosin was procured from Qualigens. Antibodies against CD11c and CD68 were purchased from InvivoGen. The corresponding fluorescent tagged secondary antibodies which were rabbit clonal were ordered from Life Science Technologies.

HT29 colon carcinoma cell line was maintained in RPMI medium with 10% FBS while THP-1 human monocytes were cultured in RPMI medium supplemented with 10% FBS, 0.05% sodium pyruvate and 0.05% β-mercaptoethanol. Both cell lines were obtained from National Center for Cell Sciences (NCCS), Pune and kept in controlled conditions at 37 °C in a 5% CO_2 incubator.

The mycelia of *P. ostreatus* was cultured in potato dextrose broth (pH 6.5) and maintained at 25–28 °C in an incubator under static conditions for 21 days. Sub-culturing of these fungal bodies was done in sterile conditions after every 3 weeks. Heteroglycans were extracted from the mycelia of *P. ostreatus* by 2% KOH alkali based method. Concisely, the mycelial biomass was first crushed in a grinder and centrifuged to obtain a pellet which was subjected to 2% KOH treatment overnight. On the very next day, centrifugation was done and the supernatant obtained was alcohol precipitated to extract the polysaccharides from the solution. The pellet obtained after centrifugation was dissolved in 20 mM Tris Buffer and purified by DEAE-Sephadex anion-exchange chromatography. Unbound flow-through was collected and lyophilized to obtain the pure heteroglycan. Biochemical tests have revealed the sample to be 90–95% pure. The heteroglycan isolated had been structurally characterized in our laboratory earlier and reported to have a high molecular weight of 2700 kDa (Devi et al., 2013).

2.2. PMA induced THP-1 differentiation

THP-1 monocytes were differentiated into macrophages after treatment with TPA (12-O-tetradecanoylphorbol-13-acetate)/PMA (phorbol-12-myristate-13-acetate) as reported earlier (Elisa et al., 2006). Morphological and marker based identification of differentiated THP-1 macrophages after PMA treatment has been described by Daigneault, Preston, Marriott, Whyte, and Dockrell (2010). Briefly, 5×10^6 cells of THP-1 were seeded to each well of a 6 well plate and incubated for 3 days with 50 ng/ml of TPA. Differentiation of monocytes into macrophages was further enhanced by replacing the TPA containing media with RPMI 1640 (10% FBS) for another 3–5 days. Morphological features of differentiated THP-1 cells have been provided in Fig. S1 (supplement file).

2.3. PolyHEMA coating of plates

600 mg of poly (2-hydroxyethyl methacrylate) or polyHEMA crystals were dissolved in 5 ml of 95% ethanol by rotating overnight (200 rpm) at 37 °C. The above solution was further diluted by adding 7 ml of 95% ethanol. 96 well plates were coated with 50 μl/well (*i.e.*, 0.1 ml/cm^2) of this polyHEMA solution and kept for drying overnight at room temperature in a sterile environment. These polyHEMA coated plates could be stored indefinitely prior to experimental use.

2.4. Viability of THP-1 cells

Cytotoxicity or viability of THP-1 cells was determined by colorimetric MTT assay. Briefly, 180 μl of THP-1 cells (adjusted to 1×10^4 cells/ml concentration) were seeded in 96 well plates and subjected to heteroglycan treatments (10, 20, 50, 100, 200 μg/ml) for 72 h. PBS was taken as control and cultures were set up in triplicates. Cells were maintained at 37 °C in a humidified 5% CO_2 incubator and MTT assay was performed at an absorbance of 595 nm to determine the cytotoxicity.