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Induction of classical activation of macrophage *in vitro* by water soluble chitin

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ABSTRACT

The purpose of this study is to understand the effect of chitin on macrophage mediated immunity, which is a significant factor to wound healing and tissue regeneration. In this work, water soluble chitin (WSC) was prepared by re-acetylation of chitosan and was treated with the murine RAW 264.7 macrophage cell lines (ATCC TIB-71). WSC induced classical activation in the RAW 264.7 cells, accompanied by the induction of associated genes. The results suggest that WSC is one of the functional chitin molecules that are responsible for the immune response, especially present in macrophage classical activation.

1. Introduction

Chitosan, the de-acetylated chitin, is widely used as an alternative of chitin, since it is known to possess a wound healing effect *via* macrophage activation. Macrophages may perform indispensable functions in infection, tissue repair, and in the resolution of inflammation in animal cells [1,2]. As a host defender, macrophages recognize and destroy foreign organisms, debride dead and damaged tissue components, and produce cytokines, growth factors, and angiogenic factors, which regulate inflammation and angiogenesis [3–6]. Macrophages are reported to have two modes of activation; classical and alternative [7]. Classical activation (M1) is induced by the combination of IFN-γ, a cytokine mainly secreted by activated Th1 T-lymphocytes and NK cells, and pro-inflammatory stimuli, which leads to up-regulation of interleukin (IL)-12, nitric oxide (NO) synthase 2 (NOS2), and a major histocompatibility complex (MHC) class II. M1 macrophages have anti-microbial and cytotoxic properties. M1 macrophages also produce pro-inflammatory chemokines that direct other inflammatory effector cells to the infected site. A number of proteins such as IL-1α, IL-1β, IL-6, iNOS, IP-10, Mip1α, Mip1β, MMP-9, MMP-12, Rantes, and TNF-α functions in M1 macrophages [1,8]. Alternative activation (M2) is driven by interleukin (IL)-4 and produces macrophages with low levels of MHC class II, IL-12, and NO [2,7]. IL-4 induces arginase activity which converts arginine to ornithine, a precursor of polyamines and collagen. This allows macrophages to participate in wound healing. Chitin, the parent molecule of chitosan (100% N-acetylglucosamine), is also known to have many useful bioactivities such as anti-fungal, and antimicrobial properties, and wound healing-promoting properties. Despite these useful properties, the application of chitin has been limited due to its poor solubility in most common organic solvents [9]. The only applicable solvents for chitin are concentrated acid (HCl, H_2SO_4, and H_3PO_4) and amide-LiCl system (N,N-dimethylacetamide-LiCl and N-methyl-2-pyrrolidone-LiCl), which cause problems such as chain hydrolysis and the removal of toxic residual solvents [10]. Many groups have attempted to modify the chemical structure of chitin to overcome these undesirable characteristics, among which the most promising method appeared to be the grafting of various monomers with hydrophilic groups onto chitin for gaining a greater water retention capacity within the grafted chitin without compromising biodegradability and bioactivity [11]. However, there are only a few reports using this method, probably due to unexpected problems. In order to solve the aforementioned problems, we used water-soluble chitin (WSC) produced by re-actylation of chitosan. Chitosan, approximately 100% DD (degree of deacetylation), was re-acetylated until the resulting chitin with a lowered DD become water-soluble. It has been reported that the lowering of the DD down to 60% made the chitosan soluble even in an aqueous solution of a neutral pH in some cases [12,13]. In our work, soluble chitin was prepared according to the report in which was shown to be obtained when the DA (degree of acetylation) is adjusted to 50–55% by re-acetylation. Here, we report that the application of WSC induced macrophage activation in the murine RAW 264.7 cell lines accompanied by induction of associated genes.

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2. Materials and methods

2.1. Materials

The medium molecular weight chitosan (Mw: 400,000, DD: 80%) was purchased from Aldrich (USA). In order to obtain highly deacetylated chitosan whose DD was over 99.03%, the chitosan was treated for 1 hr in a 47% NaOH solution at 110 °C, and the alkali treatment was repeated three times by following the method of Mima et al. [14,15]. The re-acetylation of the chitosan for manufacturing the WSC was based upon the method of Kurita et al. [12,13]. Briefly, the re-acetylation of chitosan was conducted by pouring the chitosan solution into a mixture of acetic anhydride and pyridine. In this study, 6.7-fold excess anhydride was used, and WSC (DA: 52.4%) was obtained.

2.2. Cell culture

The murine RAW 264.7 macrophage cell lines (ATCC TIB-71) were purchased from ATCC (Manassas, Virginia, USA) and maintained in a high glucose Dulbecco's modified Eagle's medium (GIBCO, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, NY, USA) and 1% penicillin–streptomycin (Invitrogen, CA, USA). Cells were incubated at 37 °C, 5% CO_2 in a humidified atmosphere. For all experiments, cells were grown to 80–90% confluency, and were subjected to no more than 10 cell passages. Subcultures were performed by scraping.

2.3. Morphological measurements

RAW 264.7 cells were seeded at 7×10^4/ml in 100 mm tissue culture dishes. After 24 h of incubation, 0.05% WSC was use to treat the culture. Cell morphology was observed every 2 h, from 0 h to 72 h after treatment. Photographs were taken using a JVC digital camera KY-F 1030U under an Olympus CKX41 microscope (10×/0.25 lens, 20×/0.40 lens, U-CMAD3 video adaptor).

2.4. RNA isolation and amplification

Total RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. After processing with DNase digestion and nucleic acid clean-up procedures, RNA samples were quantified, aliquoted, and stored at −80 °C until use. Total RNA was amplified and purified using the Ambion Illumina RNA amplification kit (Ambion, Austin, TX, USA) to yield biotinylated cRNA according to the manufacturer's protocols. Briefly, 550 ng of total RNA was reverse-transcribed to cDNA using a T7 oligo(dT) primer. Second-strand cDNA was synthesized, *in vitro* transcribed, and labeled with biotin-NTP. After purification, the cRNA was quantified using the ND-1000 Spectrophotometer (NanoDrop, NC, USA).

2.5. Microarray experiments

Microarray experiments and data analysis were carried out at Macrogen Inc. (Seoul, Korea). We performed microarray experiments using MouseRef-8 V2 Beadchip array. Total RNAs were isolated from cells and treated with WSC at seven different time points. The cDNA samples labeled 1.5 μg were hybridized with each MouseRef-8 V2 Beadchip array for 16–18 h at 58 °C, according to the manufacturer's instructions (Illumina, Inc., CA, USA). Detection of array signal was carried out using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK) as recommended in the BeadChip manual. Arrays were scanned with an Illumina Bead Array Reader confocal scanner according to the manufacturer's instructions. Array data export processing and analysis was performed using Illumina BeadStudio v3.1.3. The quality of hybridization and overall chip performance were monitored by visual inspection of both internal quality control checks and the raw scanned data. Raw data were extracted using the software provided by the manufacturer Illumina BeadStudio v3.1.3. Array data were filtered by detection p-value <0.05 (similar to signal to noise) in at least 50% of samples. All data analysis and visualization of differentially expressed genes was conducted using ArrayAssist® (Stratagene, CA, USA). Biological ontology-based analysis was performed by using Panther database (http://www.pantherdb.org).

2.6. Semiquantitative RT-PCR analysis

RNA was converted to cDNA with Oligo dT primers using the SuperScript III First-Strand Synthesis System (Invitrogen, CA, USA) according to the manufacturer's instructions. The cDNAs were amplified by the polymerase chain reaction (PCR) using the following primers listed in Table 1. PCR products were separated on a 2% agarose gel and stained with ethidium bromide.

2.7. Real-time quantitative PCR

Real-time PCR was performed using Applied Biosystems Step One Real Time PCR system (Applied Biosystems, CA, USA) with Power SYBR® Green PCR Master Mix (Applied Biosystems, CA, USA) and optical tubes according to the manufacturer's instructions. For real-time PCR, total RNA was extracted using an RNeasy mini kit and 4 μg of total RNA was reverse transcribed using a First Strand cDNA Synthesis kit. Primer sequences are listed in Table 2. Cycling parameters were as follows: initial denaturation at 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C, 30 s at 59 °C, and 30 s at 72 °C. To confirm amplification specificity, the PCR products were subjected to a melting curve analysis. Melt curve stage were 15 s 95 °C, 1 min 60 °C, and 15 s 95 °C. Negative controls containing water instead of RNA were concomitantly run to confirm that the samples were not cross-contaminated. Each reaction contained cDNA corresponding to between 80 ng of total RNA and 200 nM forward and reverse primer. Triplicate reactions were run for each sample and mRNA levels were normalized relative to β-actin.

3. Results

3.1. Effect of WSC on macrophage morphology

The Activated macrophages usually display a distinct morphology. Therefore, we examined the morphology of RAW 264.7 cells treated with WSC. Fig. 1 shows the morphological peculiarity of macrophages treated with WSC from 2 h to 72 h. Untreated RAW 264.7 cells were small, round, and contained no visible vacuoles (Fig. 1a). After 2 h, the morphology of a few RAW 264.7 cells treated with WSC had changed due to the activated morphology (Fig. 1b). After 4 h, approximately 80% of the cells showed the activated morphology with extensive cytoplasmic spreading. After 14 h, cells started to display a mature form which was larger than the control with a spindle shape (Fig. 1c). At 18 h some cells were fused and became multi-nucleated giant cells. At 38 h, cells were greatly enlarged with extensive cytoplasmic spreading.

3.2. Gene expression profiles of RAW 264.7 cells treated with WSC

We performed microarray analysis using Illumina Beadchip arrays on the RAW 264.7 murine macrophage cells treated with WSC. Comparisons of transcripts that were either up-regulated or down-regulated more than two-fold on one sample revealed that 2600 genes distinguished the cell lines treated with WSC for variable times from the untreated RAW 264.7 cells. Fig. 2 shows that