Mechanism of Macrophage Activation by Chitin Derivatives

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ABSTRACT. In order to analyze the detailed mechanisms responsible for macrophage activation by chitin derivatives, resident peritoneal macrophages were prepared and stimulated with chitin, chitosan and low-molecular weight chitosan. Our findings were as follows: (i) chitosan induced apoptosis of peritoneal macrophages, but this did not occur when chitin or water soluble low-molecular weight chitosan were used; (ii) chitosan treatment induced activation markers, such as the major histocompatibility complex (MHC) class I, class II, Fc receptors, transferrin receptor, mannose receptor, Fas, and macrophage inflammatory protein (MIP)-2, whereas chitin and low molecular weight soluble chitosan induced only the expression of MHC class I and II molecules; (iii) apoptosis induced by chitosan was mediated by the Fas signaling pathway, in response to phagocytosis via the mannose receptor. We conclude that since chitosan activates macrophages, this may be the mechanism by which it accelerates wound healing.

KEY WORDS: apoptosis, chitosan, macrophage activation, mannose receptor, wound healing.

Chitin and chitosan are polymers of N-acetyl-D-glucosamine and of D-glucosamine respectively, and which are essential structural components of the fungal cell wall. As such, these polysaccharides have been the targets of anti-fungal drugs [6]. Chitin is very similar to cellulose and they are the second most abundant polysaccharides in nature, constituting the horny substance in the exoskeletons of crabs, shrimps and insects, as well as the cell walls of infectious pathogens such as bacteria and yeast [5]. Polysaccharides and their derivatives have anticoagulant activity and are therefore potential sustained release carriers for drugs. They also accelerate wound healing and attract fibroblasts to the wound site. Moreover, they enhance neutrophil migration, induce macrophages to produce interleukin-1, tumor necrosis factor, nitric oxide and colony stimulating factor, and they have candidacidal and bactericidal activity [13–15, 17]. Indeed, chitin and chitosan have already been used to treat wounds in several countries, since they induce the rapid formation of vascular granulating tissue, the disappearance of purulence, and they promote skin regeneration with minimal scar formation [2, 5]. However, the mechanisms by which they bring about these effects are unknown.

The scope of the present study was to determine the effects of chitin derivatives on macrophages.

MATERIALS AND METHODS

Materials: Chitin and chitosan (mean diameter; 5 μm) were supplied by Sunfive Co. Ltd. (Tottori, Japan). Water soluble low molecular weight (LM-) chitosan (MW 400–20,000) was purchased from Seikakaku Kogyo Co., Ltd. (Tokyo, Japan). Mannan and laminarin were purchased from Sigma Japan (Tokyo). Mice: BALB/c, MRL-lpr/lpr, and MRL+/- mice were purchased from Japan SLC Co. (Shizuoka, Japan). The mice used in the study were approximately 10 weeks old and were housed under pathogen-free conditions in the animal facility of the Institute of Immunological Science, Hokkaido University.

Peritoneal macrophages and cell culture: Resident peritoneal macrophages were obtained by peritoneal lavage using cold phosphate buffered saline. Peritoneal cells were washed, resuspended in RPMI-1640 medium supplemented with 5% fetal calf serum, 1% L-glutamine and antibiotics (RPMI-FCS), and plated in 35 mm plastic dishes (Nalge Nunc International, Rochester, IL, U.S.A.) at 1.5 × 106 cells/ml. Adherent cell monolayers were prepared after 2 hours' incubation at 37°C in the presence 5% CO2 and washed twice with RPMI-FCS. Macrophages isolated by this procedure were over 90% pure as measured by staining for Mac 1 and B220. Chitosan, chitin or LM-chitosan were added at a final concentration of 5 or 50 μg/ml. After 6 hr, adherent cells were removed by gentle scraping with a cell scraper (Falcon 3085, BD Labware, Franklin Lakes, NJ, U.S.A.) and analyzed by flow cytometry.

Morphological analysis: Peritoneal cells were cultured in RPMI-FCS medium (1.5 × 106 cells/ml) in chamber slides (Falcon Culture Slide 4118, BD Labware) and enriched for macrophages as described previously. The cultures were then incubated with or without added polysaccharides in 5% CO2 at 37°C. After 24 hours' incubation, they were washed and stained with modified Wright Giemsa stain (Diff Quik, Harlec, Gibbstown, NJ, U.S.A.).

Antibodies, mannosylated-BSA and annexin V: PE-anti-Fas (Jo2), PE-anti-I-A4 (AMS-32.1), FITC-anti-H-2Dk (34–2–12), biotin-anti-mouse Mac1 (M1/70.15) and FITC-anti-CD45R/B220 (RA3-6B2) were purchased from PharMin (San Diego, CA, U.S.A.). FITC-anti-CD71 and transferrin receptor (R7 217.1.4) were purchased from Caltag Laboratories (Burlingame, CA).

FITC-donkey anti-rat IgG
was purchased from Jackson Immuno-Research Laboratories (West Grove, PA, U.S.A.). FITC mannosylated bovine serum albumin (BSA) was purchased from EY Laboratories (San Mateo, CA). FITC-annexin V and Red670-avidin were purchased from Kamiya Biomedical Company (Seattle, WA, U.S.A.) and Gibco BRL (Gaithersburg, MD, U.S.A.), respectively.

**Analysis by flow cytometry:** For flow cytometry analysis, cells were first pretreated with anti-FcγR mouse antibody 2.4G2 and then incubated with various antibodies followed by incubation with second-step regents. Propidium iodide (Sigma Chemical Co. St. Louis, MO, U.S.A.) was added for the last one minute to gate out dead cells. Living cells were analyzed on a flow cytometer (FACScan, Becton Dickinson, Franklin Lakes).

**Macrophage inflammatory protein (MIP)-2 assay:** MIP-2 was measured in culture supernatants with an enzyme-linked immunosorbent assay (ELISA) kit (IBL, Gunma, Japan). The sensitivity of this assay is 1 pg/ml. The measurement procedure followed an instruction manual of the ELISA kit.

**Statistical analysis:** Statistical evaluation was made by one-factor analysis of variance and Bonferroni’s multiple comparison test was applied to detect differences between groups.

**RESULTS**

Chitosan, but not chitin or LM-chitosan, induces apoptosis of resident peritoneal macrophages: Resident peritoneal macrophages were prepared from BALB/c mice and treated with chitosan, chitin, or LM-chitosan. After 24-hr, the cells treated with chitosan had aggregated around the polysaccharide and died (Fig. 1), whereas the cells treated with chitin or LM-chitosan were unaffected (Fig. 1).

To confirm that the death of the peritoneal macrophages was induced by chitosan, we performed flow cytometry analysis using propidium iodide. The results showed that chitosan induced extensive death of peritoneal cells (58.3%) within 12 hr of exposure, whereas chitin and LM-chitosan did not (20.1% and 18.6% compared to 19.4% in the control, Fig. 2A). In addition, staining with annexin V indicated that chitosan-induced cell death was due to apoptosis (chitosan: 33.3%; control: 6.3%, Fig. 2B). These findings indicate that chitosan induces apoptosis of resident peritoneal macrophages, whereas chitin and LM-chitosan do not.

Chitosan-induced activation of peritoneal macrophages: Next we examined whether chitosan induces the activation of peritoneal macrophages, using flow cytometry and ELISA. Peritoneal macrophages were cultured in media containing chitosan, chitin or LM-chitosan and analyzed for markers of macrophage activation. Chitosan induced the...

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**Fig. 1.** Chitosan-induced morphological changes in murine peritoneal macrophages. Cultures of the control (A), with 50 mg/ml chitosan (B), 50 mg/ml water soluble low molecular weight (LM-) chitosan (C), or 50 mg/ml Chitin (D) are shown.
expression of macrophage activation markers such as Fc receptors, H-2D, I-A, mannose receptor, Fas, transferrin receptor and MIP-2 (Figs. 3 and 4). These results demonstrate that chitosan induces activation of peritoneal macrophages.

Chitosan induces mannose receptor dependent Fas-mediated apoptosis of peritoneal macrophages: The Fas signaling pathway has been reported to be a major apoptosis pathway in macrophages [10]. Therefore we investigated whether the chitosan induced apoptosis was mediated by that pathway. Peritoneal macrophages were prepared from MRL-+/+ mice, and the induction of apoptosis by chitosan was assessed. As shown in Fig. 5, chitosan induced significant cell death in peritoneal macrophages derived from MRL-+/+ mice (57.5%), but not in macrophages derived from MRL-lpr/lpr mice (31.2%) (controls 18.3% and 22.6% respectively) (Fig. 5). Similar results were obtained by morphological analysis using chamber slides.

It has been reported that the mannose receptor can also bind chitosan [11]. To identify the chitosan receptor responsible for Fas-mediated apoptosis, we added mannan, which should inhibit the interaction between the mannose receptor and chitosan. As shown in Fig. 6, the chitosan-induced cell death was reduced significantly by the addition of mannan (100 mg/ml) but not of laminarin (100 mg/ml). Our findings thus indicate that the apoptosis induced by chitosan is mediated primarily by the Fas signaling pathway acting through the mannose receptor.

DISCUSSION

Chitosan and chitin have been observed to accelerate wound healing and the attainment of a satisfactory healing surface. Histological findings suggest that these substances stimulate the migration of polymorphonuclear and mononuclear cells, and accelerate connective tissue regeneration and angiogenesis [1, 12].

In the present study we have demonstrated that: (i) expression of activation markers such as Fc receptors, H-2D, I-A, mannose receptor, Fas, transferrin receptor and MIP-2 is induced by treatment with chitosan; (ii) chitosan induces apoptosis of peritoneal macrophages, but this cannot be induced by chitin and LM-chitosan; (iii) the apoptosis induced by chitosan is mediated by the Fas signaling pathway via the mannose receptor.

Since the landmark studies of Leibovich and Ross [9], showing that normal macrophage function is essential for wound healing, numerous investigators have documented the beneficial effects of macrophages on fibroblast activity and subsequent collagen synthesis [7]. Macrophage activation results in increased metabolic activity, stimulates the secretion of growth factors, cytokines, and inflammatory mediators, and enhances phagocytic activity. It is therefore reasonable to speculate that soluble factors produced by activated macrophages trigger additional cells types, such as epithelial cells and leukocytes, thus provoking an inflammatory cascade. A related observation is the induction of MIP-2 by chitosan, since Driscoll and colleagues have reported that MIP-2 stimulates epithelial cell proliferation [8]. Hence, MIP-2 production by activated macrophages may play a critical role in epithelial regeneration.

Activated macrophages are important in host immune defenses, but their uncontrolled activation can lead to septic shock and death [3, 4]. One mechanism limiting this outcome is programmed cell death, or apoptosis [18]. In our experiments, activation with chitosan was found to induce macrophage apoptosis. It took about 6 hr to induce apoptosis in 50% of the macrophages. This gradual apoptosis may
Fig. 3. Expression of activation markers on murine peritoneal macrophages. Peritoneal macrophages were cultured for 6 hr in RPMI-FCS alone (A, E, I, M, Q, and U), with 50 mg/ml chitosan (B, F, J, N, R, and V), 50 mg/ml LM-chitosan (C, G, K, O, S, and W), or 50 mg/ml chitin (D, H, L, P, T, and X). Flow cytometry was then performed using antibodies against Fc receptors (FcR; A, B, C, and D), H-2Dd (E, F, G, and H), I-Ab (I, J, K, and L), mannose receptor (mannoseR; M, N, O, and P), Fas (Q, R, S, and T), and transferrin receptor (transferrinR; U, V, W, and X). The closed area indicates staining with secondary antibody only.
be important to control wound healing acceleration and inflammation. In addition we observed the inhibition of chitosan-induced apoptosis in macrophages of MRL-lpr/lpr mice deficient in Fas-mediated apoptosis. Hence, the Fas signaling pathway seems to play an important role in chitosan-induced apoptosis.

It has been reported that the mannose receptor is the major macrophage receptor for these polysaccharides [10]. After binding to the mannose receptor, chitosan and chitin are thought to be internalized by the cell, and degraded by lysozyme to N-acetyl-D-glucosamine [16, 19]. We have demonstrated that chitosan, but not chitin and LM-chitosan, activate peritoneal macrophages, and that the action of chitosan is inhibited by mannan. Although similar, the structures of chitosan and LM chitosan differ in molecular weight and water solubility. We have observed phagocytosis of FITC labeled-chitosan particles by macrophages (data not shown). Moreover, unlike chitin, chitosan is polyanionic and can bind substances such as protein [5]. It is therefore possible that chitosan requires internalization for activation to occur, and that it exerts its effect on some cellular organelle or within the cytoplasm itself. These results suggest that further studies of chitosan in various sizes are needed to investigate whether the size of chitosan would be an important factor for the macrophage activation.

REFERENCES