PRO-APOPTOTIC EFFECT OF CHITOSAN OLIGOSACCHARIDES ON NEUTROPHILS SEPARATED FROM GLYCOGEN-INDUCED PERITONITIS IN MICE

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Abstract

To investigate the effects of chitosan oligosaccharides (COS) on neutrophils apoptosis in glycogen-induced peritonitis, DNA electrophoresis and flow cytometry were used. Neutrophils were isolated from peritonea after injection 3ml of 1% glycogen for 4h. After cultured with 100μg/ml COS for 24h, DNA were extracted and electrophoresed through 1.7% agarose gel and stained with 50μg/ml ethidium bromide. Neutrophil aopoptosis was measured by flow cytometry with the annexin-V FITC/PI apoptosis assay kit. There had no difference on DNA fragmentation in normal group, 100 μg/ml COS-treated group and 100μg/ml COS + 20μmol/L EDTA-treated group, all of them appeared DNA ladder. The 100μg/ml COS plus 1.5% butanol-treated group did not appear DNA ladder, but appeared dispersion lane. The percentage of cell apoptosis (apoptosis and late apoptosis) in 100μg/ml COS-treated group was higher than the control group after cultured for 16h. In control group, about 16% of cells displayed apoptosis after cultured for 16h, 23% of cells were apoptotic in the 100μg/ml COS-treated group. In the detection of H_2O_2 by DHR123 staining, co-cultured with COS for 22h increased the percentage of dead cells which were DHR123 positive. As an inhibitor of PLD, butanol did not change the ability of inducing H_2O_2 production by COS. However, the Ca^{2+} chelator-EDTA decreased the production of H_2O_2 stimulated by COS. At later stage of culture (32h), most of the cells were apoptosis there had no significant difference in the groups. These results showed that COS played some pro-apoptotic role in delayed apoptosis of neutrophils isolated from glycogen-induced peritonitis in mice.

Introduction

The recruitment of neutrophils from the blood stream to the venular endothelial cell surface and ultimately into the tissue interstitium constitutes a fundamental mechanism for the inflammatory response. A massive and selective increase in PMN recruitment into the peritoneal cavity was observed 4 hrs after a single intraperitoneal oyster glycogen administration. [1]. It has been reported that the total number of leukocytes present in peritoneal lavage fluid was makedly increased. This primarily reflected an 8-folded increase in neutrophils. The oyster glycogen injection is a relatively modest stimulation. Nevertheless, glycogen injection did produce an inflammatory response. Neutrophils isolated from the lavage fluid were activated, based on cell morphology and the reduced equivalents released into the extracellular space. Neutrophils undergo spontaneous apoptosis in vitro and in vivo, and it has been recognized as a crucial mechanism to elimimate the inflammation [2]. However, during the peritonitis, neutrophil numbers within tissues can be extremely high because of targeted influx from the circulation and because their constitutive

apoptotic pathway is delayed with the involvement of local inflammatory mediators. Whist neutrophils have a very short half life in the circulation (8-20h), this can increase several fold once they enter infected or inflamed tissues. In addition, inflammatory neutrophils cause tissue damage via the release of toxic reactive oxygen species and granule enzymes such as myeloperoxidase[3]. It has been reported that chitosan oligosaccharides had the ability of healing up wound in which inflammatory neutrophils played major role, this indicated that chitosan oligosaccharides had some ability of inflammation resolution. In this article, we estimated the effects of COS on the apoptosis of inflammatory neutrophils isolated from glycogen-induced peritonitis mice model.

Materials and methods

Materials

Percoll, PI, Annexin v-FITC, DHR123, RPMI1640, 1-Butanol, Oyster Glycogen, FBS (fetal bovine serum).

Animal model

Balb/c mice were injected intraperitoneally 3ml of 1% glycogen sterile solution, 4h later 1ml of PBS were injected to dilute the neutrophils in the peritonea then the mice were decaptioned and the lavage fluids were collected.

Isolation of peritoneal neutrophils

The lavage fluids were double diluted with PBS, then centrifuged for 20min at 4℃(400g), erythrocytes were removed by hypotonic lysis. The cell suspension was purified by percoll (1.089g/L) gradient. The purity of neutrophils was greater than 95% as determined by Wright-Gimesa staining. Neutrophils were suspended in RPMI1640 medium containing 10% fetal bovine serum, 100U/ml penicillin and 100μg/ml streptomycin.

DNA electrophoresis

DNA fragmentation in cultured cells was assessed by electrophoresis through agrose gels as described. DNA was extracted by CTAB method after 24-hour-incubation and was electrophoresed through 1.7% agrose gel and stained with 50μg/ml ethidium bromide and visualized under ultraviolet light[4].

Estimation of hydrogen peroxide (H_2O_2)

To assess the oxidative burst, production of H_2O_2 was estimated DHR123-labeled flow cytometry[5]. For H_2O_2 assessment, the cells were incubated with 1μmol · L^{-1} dihydrorhodamine 123 as a H_2O_2 capture for 22h. Blank control was set in which DHR123 was omitted. After incubated with DHR123, cells were harvested before an immediate detection of fluorescence intensity by flow cytometry FACS scan, and the cellular ROS level was thus measured. Propidium Iodide (PI) was used to distinguish dead cells.

Annexin v-FITC and PI double stained to assess neutrophil apoptosis

Neutrophil apoptosis was measured by flow cytometry with the annexin V-FITC/PI apoptosis assay kit[6]. The experiment was performed by following the manufacture's instructions with minor changes. After incubation, neutrophils were washed twice with ice-cold PBS and then resuspended in binding buffer. Neutrophils were analyzed by flow cytometry within 1 h of annexin V-FITC labeling. Viable neutrophils were defined as negative for annexin V-FITC and PI staining, apoptosis cells were defined as positive for annexin V-FITC but negative for PI staining, late apoptotic and necrotic cells were defined as positive for annexin V-FITC and PI staining, dead cells were defined as positive for PI staining but negative for annexin V-FITC staining.

Results and discussion

The control group, 100μg/ml COS treated group, 100μg/ml COS group and 2μM EDTA treated group displayed DNA ladder typical for apoptosis, these results were in accordance with the production of O^{2-}. 100μg/ml COS and 1.5% 1-butanol treated cells displayed dispersion lane typical for necrosis, 1-butanol has a stronger apt to inhibit the production of PA which prolongs the

life-span of neutrophils compared with the decreased production of O^{2-}, so the cells treated with 1-butanol displayed more necrosis.

When recruited into tissues, neutrophils move from normoxic to hypoxic conditions, and hence decreased O_2 tensions at inflammatory sites may extend their lifespan. However, if they are activated to secrete large quantities of reactive oxygen metabolites then this may promote their apoptosis and subsequent removal by other professional phagocytes. Neutrophils apoptosis assay were carried out after 16h-incubation, COS supplementation increased the percentage of total apoptosis (apoptosis and late apoptosis) from 16% to 23% compared with control, this alteration may be due to its effects on the production of H_2O_2. In the detection of H_2O_2 labeled with DHR123 by flow cytometry after incubation for 22hrs, PI staining was used to distinguish dead cells. The control group appeared 34.27% DHR123 positive and PI negative, 51.16% DHR123 and PI positive; incubation with 100μg/ml COS for 22h resulted in 10.88% DHR123 positive and PI negative, 75.69% DHR123 and PI positive. These results suggested that COS stimulated the production of H_2O_2 which then promoted more cells die via apoptosis. About 22.35% of the neutrophils appeared DHR123 positive and PI negative, 67.55% of the neutrophils appeared DHR123 and PI positive after butanol administration. Butanol inhibited the production of PA by PLD/PA pathway, PA has the ability of delaying apoptosis of neutrophils, so the decrease of PA resulted in the decrease of percentage of viable cells from 34.27% to 22.35%. When co-incubated with COS and butanol the percentages of DHR123 positive viable cells and DHR123 positive dead cells had no significant change controlled with in comparison with COS treated cells, which meant that COS stimulated the production of H_2O_2 might not be through PLD/PA pahway. 20μM EDTA inclusion led to lower percentage of DHR123 positive and PI negative cells (from 34.27% to 19.7%), same level of DHR123 and PI positive cells (from 51.16% to 56%), higher percentage of DHR123 negative and PI positive cells (from 8.06% to 19.6%), these findings indicated that chelating of Ca^{2+} resulting in more dead cells but not through the production of H_2O_2. When co-incubated with EDTA and COS, the cells displayed higher percentage of DHR123 and PI positive cells than EDTA-treated groups (from 56% to 67.24%). These results implicated that the ability to produce H_2O_2 by COS stimulation was Ca^{2+}-dependent. At later stage of co-incubation for 32h, the percentage of apoptosis between the control and COS-treated cells had no difference (from 81% to 84%), this may be because at this time most of the neutrophils executed apoptosis or necrosis.

In conclusion, COS treatment promoted the delayed apoptosis of inflammatory neutrophils through stimulating the production of H_2O_2. Furthermore, it might result in the resolution of inflammatory response and easement of tissue injury.

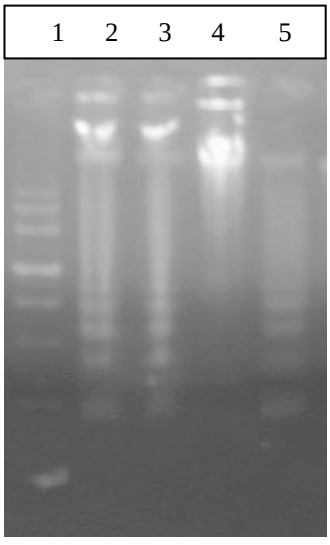

Figure 1 : DNA samples extrated from neutrophils cultured for 24h with medium alone (lane 2), 100μg/ml COS (lane3), 100μg/ml COS plus 1.5% 1-butanol (lane 4), 100μg/ml COS plus 20μM EDTA (lane 5) were eletrophoresed through 1.7% of agarose gel as described in Materials and Methods.

Fig 2 A B

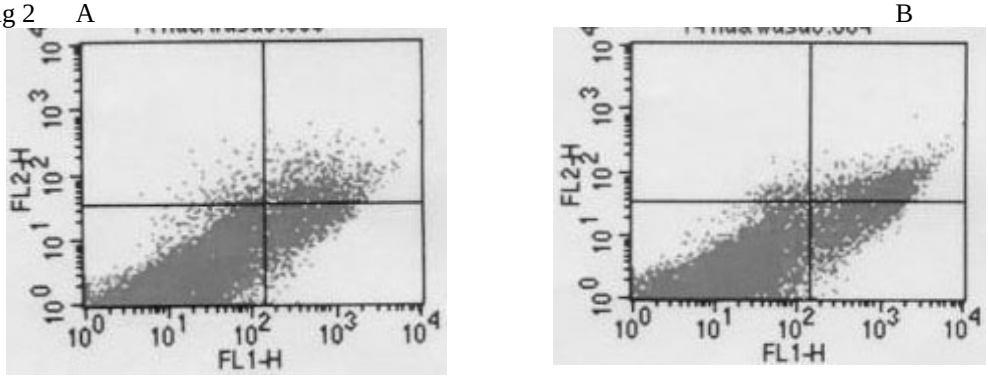

Figure 2 : Detection of neutrophils apoptosis using Annexin v-FITC and PI staining by flow cytometry after cultured for 16h. A: neutrophils cultured with medium alone, B: neutrophils cultured with 100μg/ml COS.
FL 1-H: Annexin v-FITC; FL 2-H: PI.

Fig 3 A B C D E F

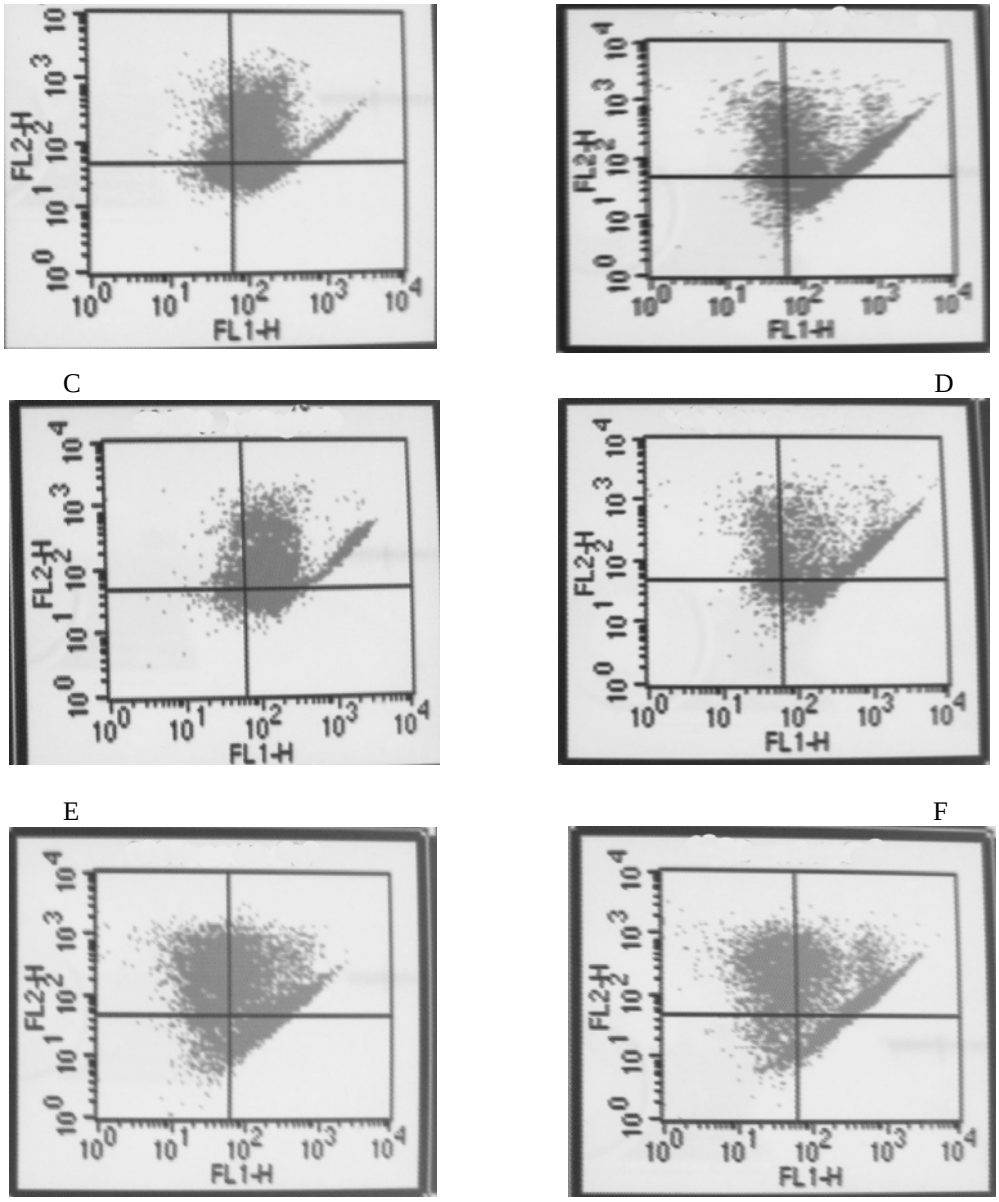

Figure 3 : Determination of H_2O_2 production using DHR123 by flow cytometry after cultured for 22h, PI staining was used to distinguish died cells. A: neutrophils cultured with medium alone, B: neutrophils cultured with 100μg/ml COS, C: neutrophils cultured with 1.5% 1-butanol, D: neutrophils cultured with 100μg/ml COS plus 1.5% 1-butanol, E: neutrophils cultured with 20μM EDTA, F: neutrophils cultured with 100μg/ml COS plus 20μM EDTA.
FL 1-H: DHR123; FL 2-H: PI.

Fig 4 A B

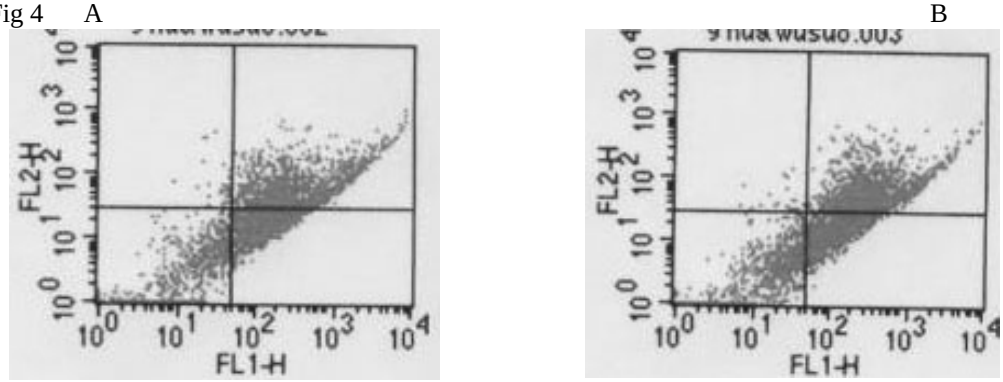

Figure 4 : Detection of neutrophils apoptosis using Annexin v-FITC and PI staining by flow cytometry after cultured for 32h. A: neutrophils cultured with medium alone, B: neutrophils cultured with 100μg/ml COS.
FL 1-H: Annexin v-FITC; FL 2-H: PI

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