ACTIVATION OF MURINE MACROPHAGES BY GRIFOLAN

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Abstract

A gel-forming (1→3)-β-D-glucan, grifolan (GRN) from edible mushroom, enhances various immunological activities. In this paper, effect of GRN on the induction of cytokines and nitric oxide by macrophage (MP) cell line (RAW264.7), peritoneal MP (PM) and kupffer cell would be shown. GRN bound to MP was detected immunohistochemically using anti-GRN antibody. GRN could induce production of TNFα, IL-1α, and IL-6 by RAW264.7. MP also induced those cytokines by incubation with GRN. GRN induced phosphorylation of MAP kinase and p38 of PM.

The kinetic study on the activation of kupffer cells revealed that GRN could induce enhanced production of cytokines and nitric oxide on 4 to 7 days after *i.v.* administration of GRN. Cytostatic activity of kupffer cells against murine lymphoma, EL-4, was also augmented by GRN with similar time course to nitric oxide production. The cytostatic activity was dependent on nitric oxide, since iNOS inhibitor diminished the cytostatic activity. Administration of GRN increased expression of CD11b, known as a β-glucan receptor, on kupffer cells on day 7. Above data suggested that GRN could activate murine MPs to enhance production of cytokines and nitric oxide.

Introduction

Grifolan (GRN) is a gel-forming (1→3)-ß-D-glucan from *Grifola frondosa* and is known as an immunopotentiator having host-mediated antitumor activities (Yadomae, T. and N. Ohno. 1996). One of the most important activation mechanisms of ß-glucans on host defense system is enhancement macrophage function (Ohno et al., 1986). Macrophage is known as an important cell for providing initial host defense signals by producing various cytokines and cytotoxic effect on tumor cells (Nathan DF. 1987). In case of peritoneal macrophages (PM), GRN had an ability to induce some inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1α (IL-1α), and interleukin-6 (IL-6) (Adachi et al., 1994). It was shown that neutrophils would be accumulated in tissues where GRN was distributed in initial period (Yadomae, 1992). In spite of a hypothesis that GRN could induce chemotactic factors, it is unclear what kinds of chemokines are induced by stimulation with GRN. Signaling pathways induced by GRN also investigated with tyrosine phosphorylation of MAP kinase family of peritoneal macrophages.

Furthermore, cytostatic activity of PM against murine tumor cell was enhanced by GRN-administration.(Yadomae, 1992) It has been clarified that most ß-glucans are distributed to reticuloendothelial system, such as liver and spleen, in mouse after systemic administration (Suda et al.,1996). However little is known concerning an effect of GRN on function of kupffer cells. Therefore, we focused on examination of kupffer cells' activity induced by administration of GRN. To examine several activities of kupffer cells, production of cytokines and nitric oxide and cytostatic activity of kupffer cells were tested. Present paper will report activation of several function of kupffer cells by GRN administration.

Results and Discussion

Induction of Neutrophil Chemotactic Factor

We examined whether ß -glucans possess an ability to induce chemotactic factor of macrophages. Peritoneal macrophages were cultured with (1→6)-branched (1→3)-ß-D-glucans such as GRN, SPG, and SSG, or particulate ß-glucans, zymosan, as stimuli. As shown in Fig. 1, the neutrophil chemotactic activity in the supernatant was observed only in GRN and zymosan, whilst SPG and SSG had no ability to induce chemotactic activities. This productivity in chemotactic factor was consistent with other inflammatory cytokines such as TNFα.

Northern Blotting of Murine KC

To identify factors for neutrophil chemotaxis in activation with GRN, northern blotting was done using murine KC cDNA, which codes neutrophil chemokine. As shown in Fig. 2, significant increase of KC mRNA was observed by stimulation with GRN at 1 h and 6 h compared with medium alone. This suggests that elevated KC mRNA expression persists in the presence of GRN.

Enzyme Immunoassay for Chemokine in Culture Supernatant

It is reported that murine KC is highly homologous to rat cytokine-induced neutrophil chemoattractant (CINC) (Watanabe et al., 1989). A commercially available rat CINC enzyme immunoassay (EIA) was applied to detect murine KC. CINC released in the supernatant was elevated by culturing peritoneal macrophages with 50 to 500 μg/ml GRN for 3 to 48 h (Fig.3). Taken together, results of Fig. 2 and 3 suggest that KC is a candidate owing to the neutrophil chemotactic factor in the supernatant.

Tyrosine Phosphorylation of MAP Kinase Family

In order to estimate signal transduction pathway induced by GRN, tyrosine phosphorylation of MAP kinase family was examined. Peritoneal macrophages were cultured with various β-glucans for 0 to 45 min and lysed to obtain soluble cellular proteins. The proteins were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels, and blotted on

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a membrane. The proteins on the membrane were visulalized by staining with antibody specific to phosphorylated ERK1 and ERK2. As shown in Fig. 4, proteins around 42 and 44 kDa were detected in samples from zymosan-treated cell lysate and GRN-treated one. However, SPG and laminarin had no such ability to phosphorylate those proteins. In addition to ERK, phosphorylation of p38, another member of MAP kinase family, was examined by combination of immunoprecipitaion of cell lysate with anti-phosphotyrosine antibody and western blotting with anti-p38 antibody, respectively. Phosphorylation of p38 was observed in not only zymosan-treated lysate but also in GRN-treated one. Futhermore, LPS also induced phosphorylation of p38. These results suggest that MAP kinase families of ERK-1, ERK-2 and p38 are activated by stimulation with GRN as well as that with zymosan. These are implying that activation pathway by GRN is similar to that by LPS (Sanghera et al.,1996).

Localization of GRN in Liver

As described in Introduction, β-glucans administered in host are liable to accumulate into reticuloendothelial systems. We examined what kind of cells are involved in localization of GRN. A frozen liver section from ICR mouse was made on day 7 after *i.v.* administration of GRN, and was immunochemically stained using anti-GRN antibody. As shown in Fig. 6, GRN was detected as associating with non-parenchymal cells in liver. The results suggest that kupffer cells are likely to bind to GRN in liver. To investigate this possibility, we have isolated kupffer cells after administration of GRN on day 1, 4 and 7. Several biological activity of kupffer cells were tested with productivity of cytokines and nitric oxide.

Production of Cytokines by Kupffer Cells

Production of interleukin 1α and interleukin 6 in cell lysate and supernatant, respectively, was augmented after *i.v.* administration of GRN (Fig. 7 and 8). The cytokine production was further enhanced by adding GRN or LPS in vitro. However, there was no detectable TNFα production in the culture supernatant tested. This result agrees with other report that TNFα productivity of kupffer cells is lower than peritoneal macrophages (Kawada et al., 1992).

Nitric Oxide Production by Kupffer Cells

Nitric oxide (NO) released in culture supernatant was also monitored after administration with GRN. Amounts of NO was increased in accord with days after administration of GRN (Fig. 9).

Cytostatic Activity of Kupffer Cells against EL-4 Lymphoma

Peritoneal macrophages are reported to be activated as cytostatic against tumor cells after administration of GRN. Therefore, cytostatic activity of kupffer cells also investigated. As shown in Fig.10), The activity was increased in accord with days after administration of GRN. The activity showed dependency of effector cell ratio.

Contribution of Nitric Oxide to Cytostatic Activity of Kupffer Cells

NO is known as a effector molecule on tumor cell destruction, effect of NO synthase inhibitor on cytostatic activity of kupffer cells was examined. As shown in Fig. 11, kupffer cells on day 7 showed significant cytostatic activity. The activity was completely reduced to control level by adding of N-mono methyl arginine (NMMA) to culture. NO release in supernatant was also reduced by adding NMMA (Fig. 12). These results clearly indicate that the cytostatic activity of kupffer cells is resulting from elevated production of NO.

Effect of GRN on Expression of CR3 on Kupffer Cells

To elucidate why kupffer cells on day 7 showed higher response to further addition of GRN, we tried to examine expression level of CR3 which is thought to act as β-glucan receptor (Thornton et al. 1996). As shown in Fig. 13, CR3 expression on kupffer cells were increased on day 7 after *i.v.* administration of GRN, although the cells on day 1 showed low level. These results suggest that administration of GRN up-regulate CR3 expression to render the cells responsive to additional GRN.

Conclusion

GRN could activate extensively peritoneal macrophage function through phosphorylation of MAP kinase. The activation was indicated by production of cytokines including neutrophil chemoattractant. Administration of GRN also affect various biological activity of kupffer cells. The activities of kupffer cells induced by GRN are similar to those of other local tissue macrophages. We have found that cytostatic activity of kupffer cells against EL-4 lymphoma is closely related to productivity of nitric oxide. Enhanced sensitivity of kupffer cells to GRN might be explained by increased level of CR3, a candidate for β-glucan receptor.

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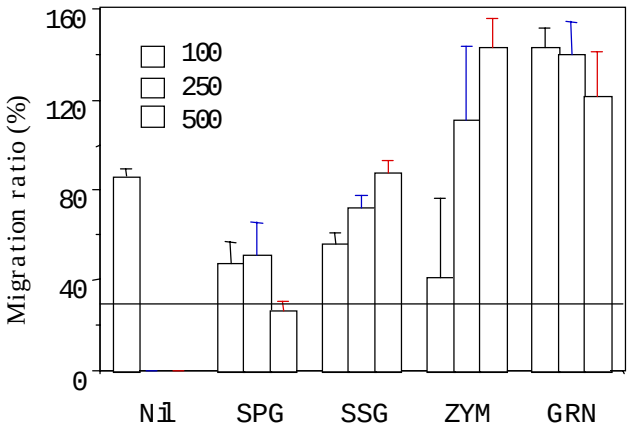

Figure 1. 1 Induction of Neutrophil Chemotactic Factor by Peritoneal Macrophages.

PEM was culture with various bE-glucans at indicated concentration (μg/ml) for 24 h at 37g/C, then the supernatants were applied to chemotaxis assay. Horizontal line shows migration ratio of neutrophil toward medium alone.

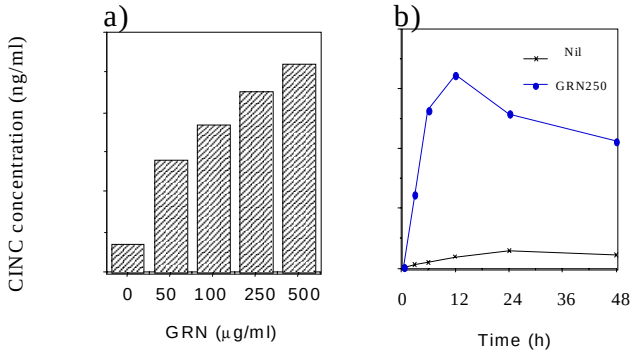

Figure 2. GRN Can Induce Chemotactic Factor Reactive to Rat CINC ELISA System.

a) Peritoneal macrophages were incubated with various concentration of GRN for 24h at 37C. Anti-rat CINC antibody reactive products in the culture supernatant was measured by ELISA.

b) Kinetic assay of anti-rat CINC antibody reactive products.

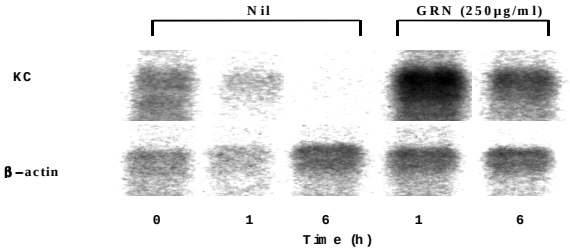

Figure 3. Northern Blotting Analysis for Murine KC in Peritoneal Macrophages.

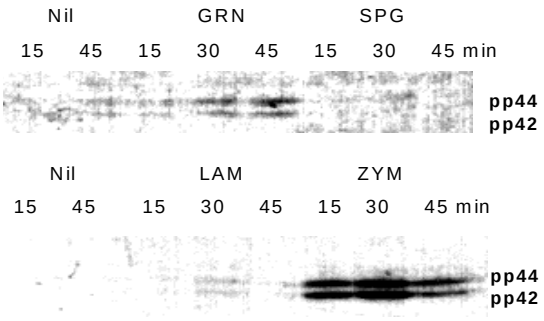

Figure 4. Phosphorylatin of MAP Kinase (ERK-1 and ERK-2) in Macrophage Lysate.

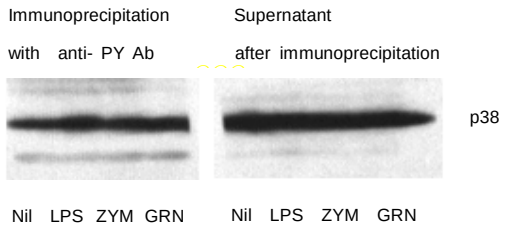

Figure 5. Phosphorylation of p38 Induced with Various Stimuli.

Peritoneal macrophages were cultured with stimuli for 15min at 37ºC. Cell lysates were analyzed by western blotting using mouse anti-phosphotyrosine antibody and anti-p38 antibody.

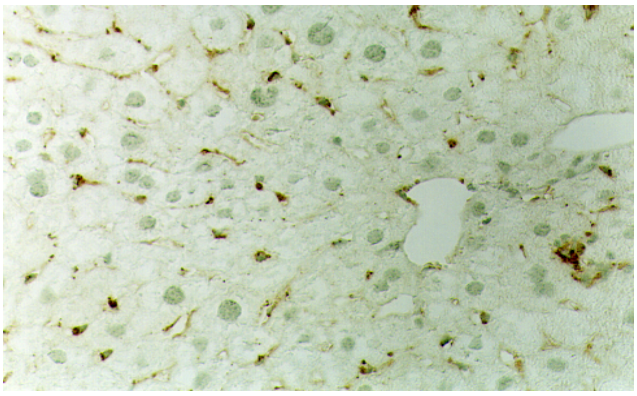

Figure 6. Localization of GRN in Liver.

GRN in liver was detected by anti-GRN antibody after intravenous injection of GRN (250μg/ml). Magnification : x 400.

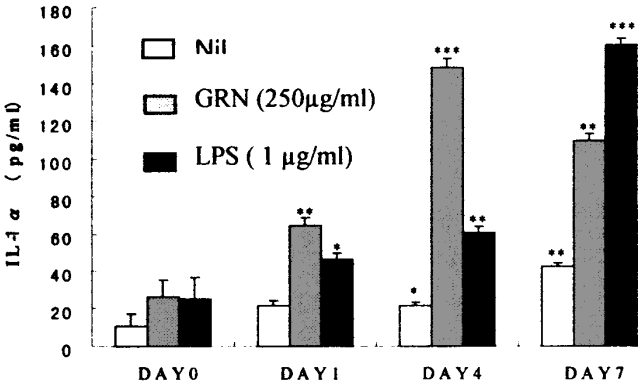

Figure 7. IL-1α Production by Kupffer Cells Obtained from GRN-Administered Mice.

Kupffer cells isolated on day 0, 1, 4, and 7 after administration with GRN were cultured for 24h at 37ºC in the presence of 250 μg/ml GRN or 1 μg/ml lipopolysaccharides. Each cell lysate was prepared repeated freezing and thawing. IL-1α content was determined using enzyme linked immunosorbent assay. Statistical significance against day 0 was determined by Student's method * ; p < 0.05, ** p< 0.01, *** p<0.001. Six mice in each group were used to obtain data. Error bars represent standard deviation (SD).

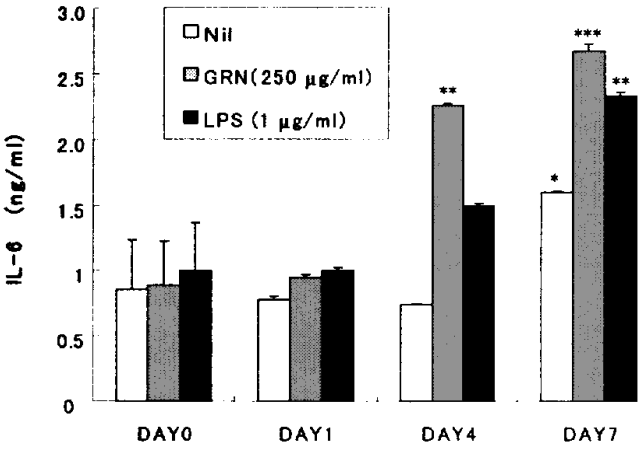

Figure 8. IL-6 Production by Kupffer Cells.

IL-6 content in each culture supernatant was determined by ELISA. Statistical significance against day 0 was determined by Student's method. * p< 0.05, ** p< 0.01. *** p<0.001. n=6.

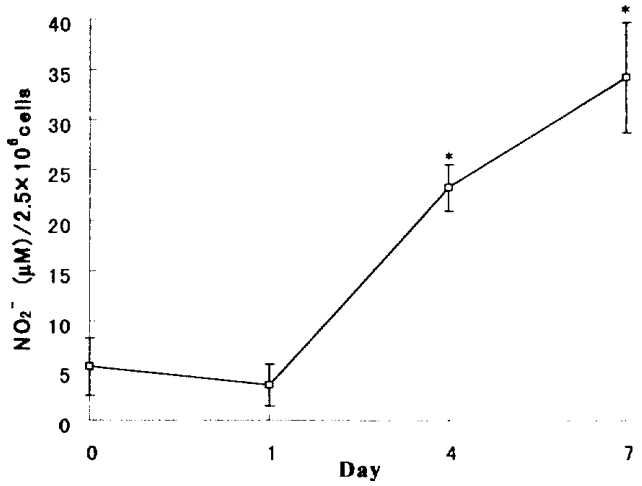

Figure 9. Nitric Oxide Production by Kupffer Cells Obtained from GRN-Administered Mice.

Kupffer cells isolated on day 0, 1, 4, and 7after administration with GRN were cultured for 24h at 37ºC. Nitric oxide content in the culture supernatant was determined using Griess. Statistical significance against day 0 was determined by Student's method * p< 0.01. n=6

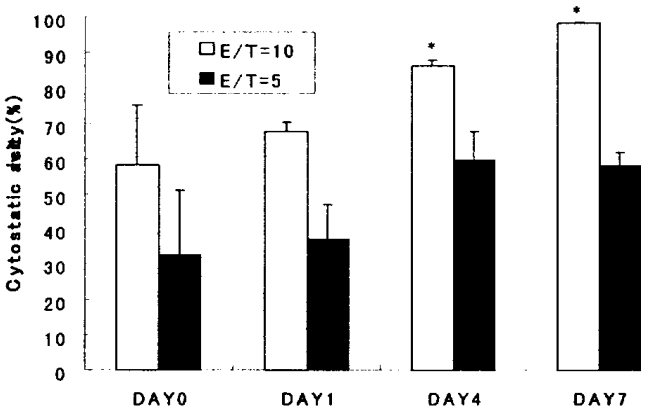

Figure. 10 Cytostatic activity of kupffer cells against EL-4 lymphoma.

Kupffer cells (5×10^5 cells) isolated on day 0, 1, 4, and 7after administration with GRN were cultured with EL-4 cells (■; 1×10^5cells or □; 5×10^4 cells) for 44h at 37ºC in 96 well culture plate. To assess proliferation of EL-4 cells, WST-1 was added to the culture plate and further incubated for 4 h at 37ºC.*$p<0.05$. n=3.

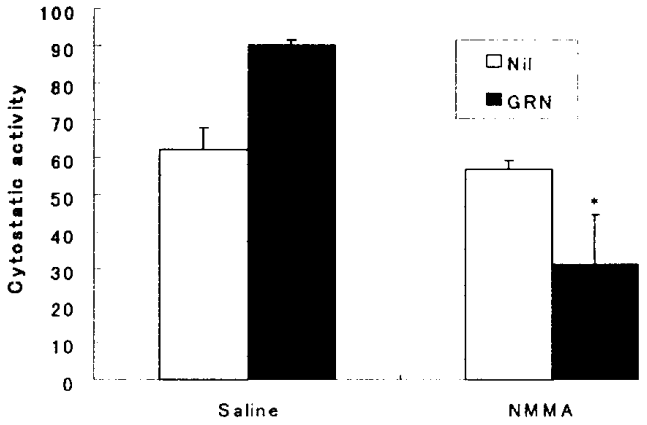

Figure 11. Effect of NOS Inhibitor on Cytostatic Activity of Kupffer Cells.

Kupffer cells on day 7 after GRN- or saline-administration were cultured with EL-4 as described in Fig. 5 at effector/target ratio = 10/1 in the presence of saline (□) or 25 μg/ml of NMMA (■). Cytostatic activity was calculated as described in Materials and Methods. Significance to saline-treated group; * $p<0.01$. Error bars represent SD.

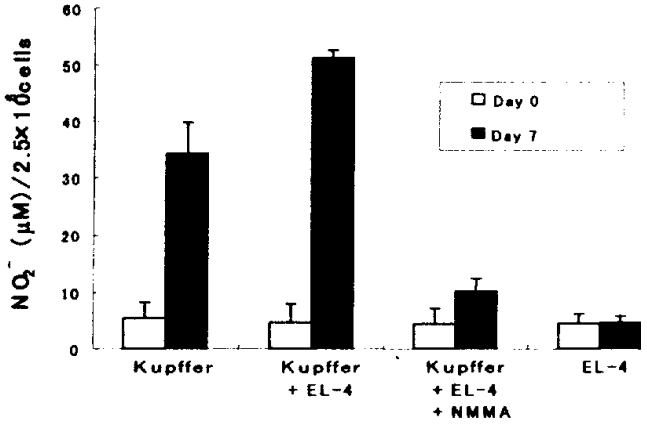

Figure 12. Nitric Oxide Production by EL-4 and Kupffer Cells Obtained from GRN-Administered Mice.

Kupffer cells isolated on day 0 (□) and 7 (■) after administration of GRN were cultured for 48h at 37ºC with or without EL-4 lymphoma and 25 μg/ml NMMA as indicated. Nitric oxide content in the culture supernatant was determined using Griess reagent as described in Materials and Methods. Statistical significance to the absence of NMMA was determined by Student's method * $p< 0.001$. Error bars represent SD.

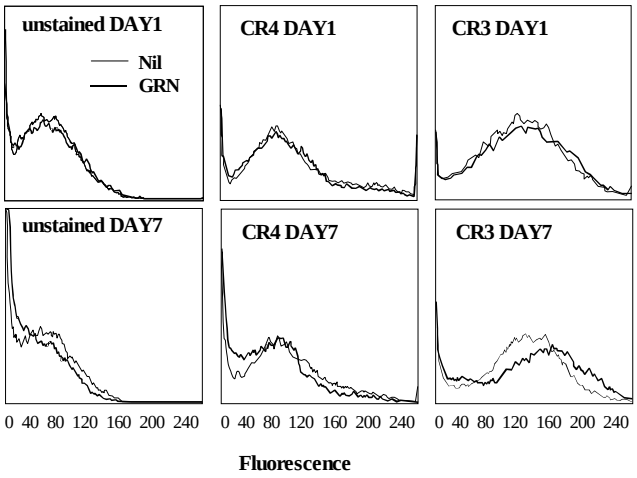

Figure 13. Flow Cytometry of Kupffer Cells Obtained from GRN-Administered Mice.

Kupffer cells isolated on day 1 and 7 after administration of GRN were treated with FITC-conjugated anti-CR3 or CR4 monoclonal antibody. Histgram of cell fluorescence was composed of 10000 cells in each panel.