The concentrations of GM-CSF, IL-10, and IL-12p70 were determined using OptEISA™ kit (PharMingen).

RT-PCR

Total cellular RNA was extracted from the cell suspension using Isogen (Wako). The RNA fraction was dissolved in 20 μl DEPC-treated water. cDNA was prepared using oligo dT primer and Moloney murine leukemia virus reverse transcriptase (MMLV RT) (Promega, Madison, WI). Samples were stored at 4°C until use. Sample cDNAs were amplified in the presence of sense primer, antisense primer, and premix in PCR buffer. PCR was done in a TaKaRa PCR Thermal Cycler MP for 25 cycles, each cycle consisting of 30 sec denaturation at 94°C, 30 sec annealing at 60°C, and 40 sec extension at 68°C. Reaction products were made visible by electrophoresis of 5 μl reaction mixture at 100 V for 20 min. in a 1.2 % agarose gel. The primers used for this study were GAPDH, sense primer (ss), 5'-CCATCACCATCTTCCAGGAG-3', antisense primer (aa), 5'-CCTGCTTCACCACCTTCTTG-3'; GM-CSF ss; 5'-GGCCTTGGAAGCATGTAGAG-3', aa, 5'-TCCGCTGTCCAAGCTGAGT-3'.

Fractionation of spleen cells for cell culture

Splenocytes were removed from mice. The spleen was teased apart in RPMI 1640 medium, and after centrifugation, the single cell suspension was treated with ACK-lysing buffer to lyse red blood cells. After centrifugation, splenocytes were maintained in RPMI 1640 medium supplemented with 50 μg/ml gentamicin sulfate and 10% FBS. For preparation of adherent cells, spleen cell suspensions (1×10^7 cells/ml) were incubated in plastic 24-well flat-bottomed plates at 37°C for 1.5 h in a humidified 5% CO_2/95% air atmosphere. After incubation, nonadherent cells were collected, and the plate was washed with RPMI 1640 medium prewarmed at 37°C. After centrifugation, the nonadherent cells were suspended in 10% FBS/RPMI medium and applied to nylon wool columns (Wako). The column-passed fraction (CPF) was used as the T cell-enriched fraction.

Statistical analysis

The results were expressed as the means ± standard deviation (SD). The significance of differences between the means was measured by Student's *t*-test.

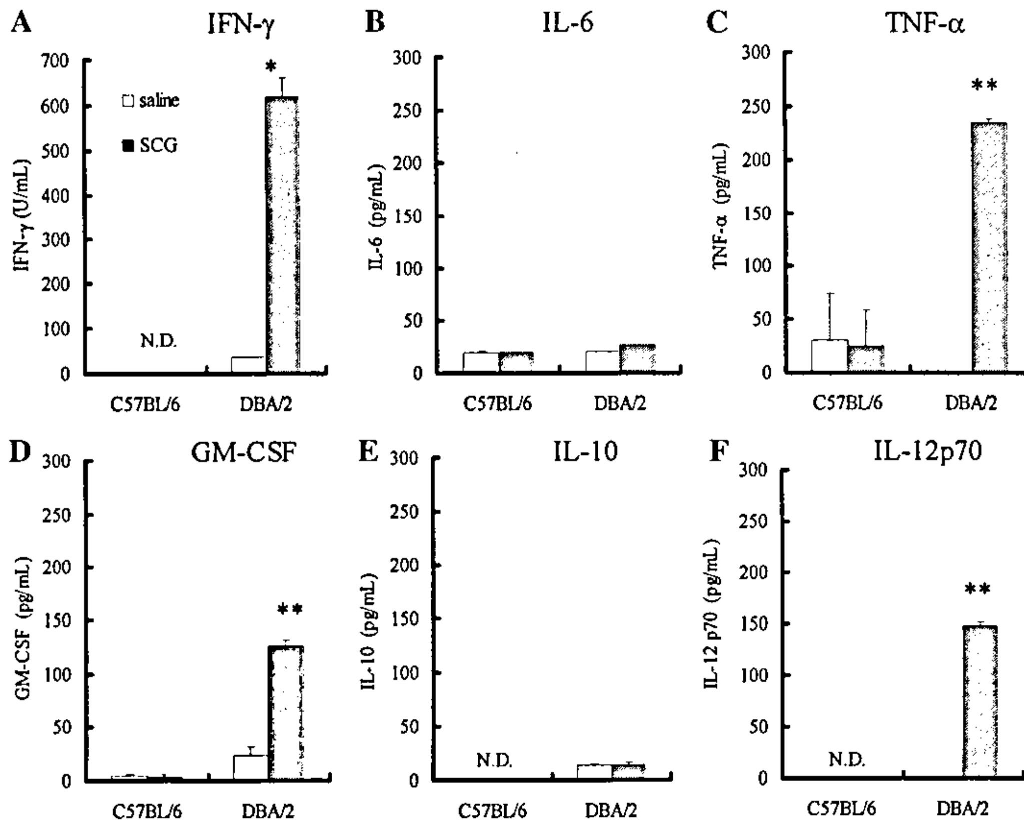

FIG. 1. Cytokine production by splenocytes stimulated with SCG in C57BL/6 and DBA/2 mice by ELISA. Splenocytes in C57BL/6 and DBA/2 mice were isolated. Cell suspensions were adjusted to 5×10^6 cells/ml in 10% FBS/RPMI medium and incubated for 48 h in the presence or absence of SCG (100 μg/ml). After incubation, the supernatant was collected. The concentrations of (**A**) IFN-γ, (**B**) IL-6, (**C**) TNF-α, (**D**) GM-CSF, (**E**) IL-10, and (**F**) IL-12p70 were determined by ELISA. The data show one of at least three experiments performed, with similar results, each evaluating two mice per group. Values represent the means ± SD. Significant difference from control, $*p < 0.05$, $**p < 0.01$.

RESULTS

Effect of SCG on cytokine synthesis in DBA/2 and C57BL/6 mice

We previously reported that IFN-γ production was not induced by SCG in splenocytes derived from inbred strains of mice except for DBA/1 and DBA/2 mice. This suggested that splenocytes derived from DBA/1 and DBA/2 mice were sensitive to SCG *in vitro* and released IFN-γ by SCG. To clarify the mechanism responsible for this, cytokines induced by SCG in splenocytes from DBA/2 mice were screened by the ELISA system. Splenocytes from DBA/2 or C57BL/6 mice were cultured in the presence or absence of SCG (100 μg/ml) *in vitro* to collect the supernatant. The concentrations of IFN-γ, IL-6, TNF-α, GM-CSF, IL-10, and IL-12p70 were measured. As shown in Figure 1, in DBA/2 mice, the concentrations of IFN-γ, TNF-α, GM-CSF, and IL-12p70 were significantly increased, but IL-6 and IL-10 production was not induced by SCG. However, all cytokines measured were not induced by SCG in splenocytes from C57BL/6 mice.

Next, cytokines induced by SCG in splenocytes from DBA/2 mice were screened by the CBA system. The concentrations of TNF-α and IFN-γ were significantly higher due to SCG, but production of IL-5, IL-4, and IL-2 was not induced by SCG (date not shown). SCG responded to the splenocytes from DBA/2 mice to induce IFN-γ, TNF-α, GM-CSF, and IL-12p70.

To confirm the effect of SCG on cytokine synthesis in C57BL/6 mice, splenocytes from C57BL/6 mice were cultured with SCG (200, 100, 50, 10, 1, or 0 μg/ml) *in vitro* for 48 h to collect the supernatant. Cytokines in the supernatant were measured, and none of cytokines were induced by SCG (data not shown). In addition, cytokines were not induced by SCG (100 μg/ml) after 12, 24, 48, or 72 h of culture (data not shown).

Effect of cytokines on SCG-induced cytokine production in C57BL/6 mice

To investigate the key cytokine associated with cytokine synthesis induced by SCG, splenocytes from C57BL/6 mice were cultured with SCG (100 μg/ml) or lipopolysaccharide (LPS) (100 ng/ml) in the presence of exogenous cytokines, such as rMuGM-CSF, rMuIFN-γ, rMuIL-12p70, or rMuTNF-α (1 ng/ml) *in vitro*. As shown in Figure 2E, by exogenously adding rMuGM-CSF to the splenocyte culture system, the concentrations of IFN-γ, TNF-α, and IL-12p70 were significantly in-

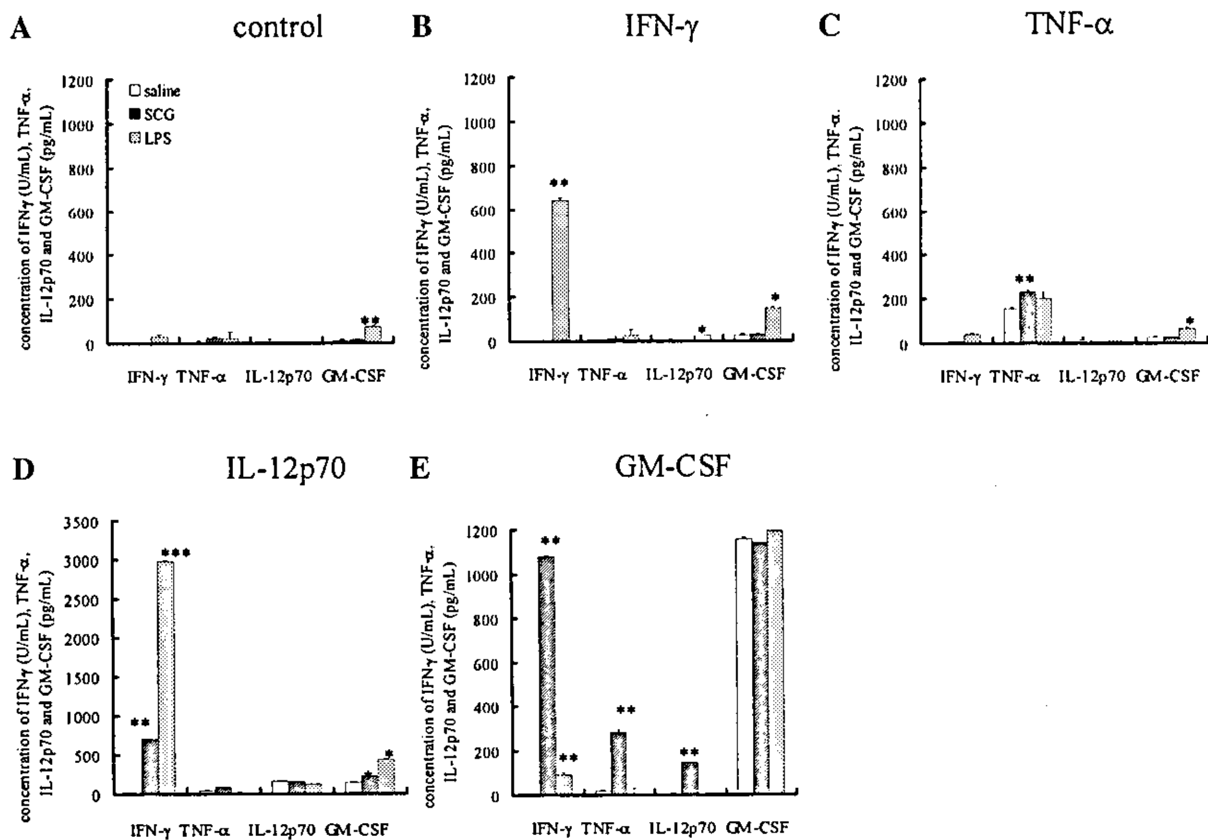

FIG. 2. Cytokine production by splenocytes stimulated with SCG in the presence of cytokines in C57BL/6 mice. Splenocytes in C57BL/6 mice were isolated and cell suspensions were adjusted to 5×10^6 cells/ml in 10% FBS/RPMI medium. (**A**) Control. Splenocytes were incubated for 48 h with SCG (100 μg/ml) in the presence of (**B**) rMuIFN-γ, (**C**) rMuTNF-α, (**D**) rMuIL-12p70, or (**E**) rMuGM-CSF (1 ng/ml). After incubation, the supernatant was collected. The concentration of IFN-γ, TNF-α, IL-12p70, and GM-CSF in the supernatant was determined by ELISA. The data show one of three experiments performed, with similar results, each evaluating two mice per group. Values represent the means ± SD. Significant difference from control, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

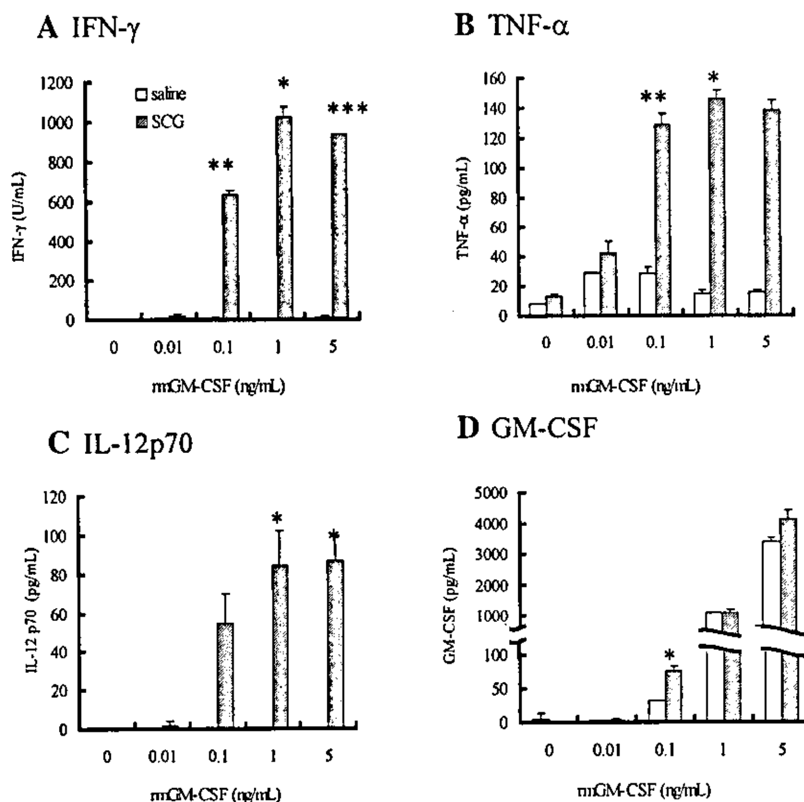

FIG. 3. Dose-response of rMuGM-CSF on cytokine production by splenocytes stimulated with SCG. (**A**) Splenocytes in C57BL/6 mice were isolated, and cell suspensions were adjusted to 5×10^6 cells/m in 10% FBS/RPMI medium. They were incubated for 48 h with SCG (100 μg/ml) in the presence of rMuGM-CSF (5, 1, 0.1, 0.01, or 0 ng/ml). After incubation, the supernatant was collected. The concentrations of (**A**) IFN-γ, (**B**) TNF-α, (**C**) IL-12p70, and (**D**) GM-CSF in the supernatant were determined by ELISA. The data show one of three experiments performed, with similar results, each evaluating two mice per group. Values represent the means ± SD. Significant difference from the control, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

creased due to SCG in C57BL/6 mice. Cytokine production induced by SCG in the presence of GM-CSF was higher than that induced by LPS. These results suggested that GM-CSF was the key cytokine. As shown in Figure 2D, the exogenous addition of IL-12 to the splenocyte culture system augmented IFN-γ induction by SCG as well as LPS. However, IL-12 could not induce TNF-α by SCG under these experimental conditions. The exogenous addition of IFN-γ or TNF-α to the splenocyte culture system did not produce any effect on induction of the other cytokines by SCG (Fig. 2B,C).

We next examined dose-responses of GM-CSF in cytokine induction by SCG in C57BL/6 mice. Splenocytes from C57BL/6 mice were cultured with SCG (100 μg/ml) in the presence of rMuGM-CSF (0, 0.01, 0.1, 1, or 5 ng/ml) *in vitro* to collect the supernatant. The concentrations of IFN-γ, TNF-α, IL-12p70, and GM-CSF were increased by SCG in a dose-dependent manner (Fig. 3). The minimum dose of GM-CSF exogenously added to the culture system on cytokine induction by SCG was 0.1 ng/ml. To confirm that the exogenous addition of GM-CSF enhanced the endogenous GM-CSF by SCG, the effect of SCG on the gene expression of GM-CSF mRNA in the splenocytes was analyzed by RT-PCR. Splenocytes from C57BL/6 mice were cultured with SCG (100 μg/ml) in the presence of rMuGM-CSF (1 ng/ml) *in vitro* for 36 h. Total RNA was prepared by Isogen. Samples of RNA preparation were reverse transcribed to cDNA and run on PCR using a set of GAPDH primer to monitor quality and quantity of RNA preparation. After approval of the RNA preparation, GM-CSF mRNA expression was compared. The gene expression was compared by densitometry of the PCR products on each agarose gel. To compare the quantity of gene expression, different volumes of cDNA template were run on PCR. As shown in Figure 4, SCG stimulation enhanced expression of GM-CSF in 36 h. These results

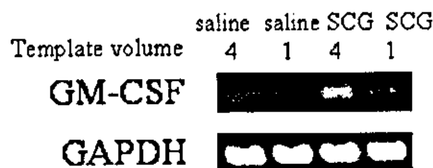

FIG. 4. mRNA expression of GM-CSF in the splenocytes from C57BL/6 mice. Splenocytes in C57BL/6 mice were isolated, and cell suspensions were adjusted to 5×10^6 cells/ml in 10% FBS/RPMI medium. They were incubated for 36 h with SCG (100 μg/ml) in the presence of rMuGM-CSF (1 ng/ml). RT-PCR of GAPDH and GM-CSF was performed as described in Materials and Methods.

suggested that GM-CSF elicited SCG-induced IFN-γ, TNF-α, IL-12p70, and GM-CSF.

Effect of GM-CSF on SCG-induced cytokine production in DBA/2 mice

We next examined the effect of rMuGM-CSF or anti-GM-CSF antibody added to the splenocyte culture system in DBA/2 mice. First, splenocytes from C57BL/6 or DBA/2 mice were cultured with SCG (100 μg/ml) in the presence of rMuGM-CSF (1 ng/ml) *in vitro* to collect the supernatant. As shown in Figure 5, the exogenous addition of GM-CSF in C57BL/6 mice synergistically enhanced the induction of other cytokines by SCG. However, such synergistic effect was not seen in DBA/2 mice. Therefore, the concentration of endogenous GM-CSF was sufficient to promote the cytokine induction of SCG in DBA/2 mice.

Splenocytes from DBA/2 mice were cultured with SCG (100 μg/ml) in the presence of anti-GM-CSF mAb (0, 0.1, 0.25, or 0.5 μg/ml) *in vitro* in order to collect the supernatant. Neutralizing GM-CSF using anti-GM-CSF mAb significantly inhibited IFN-γ, TNF-α, and IL-12p70 elicited by SCG in a dose-dependent manner (Fig. 6A). However, the addition of high-dose anti-GM-CSF mAb to the splenocyte culture system in DBA/2 mice had no influence on cytokine induction by LPS (Fig. 6B). These results confirmed our hypothesis about the importance of endogenous GM-CSF in the induction of cytokine synthesis by SCG in DBA/2 mice.

Effect of GM-CSF on SCG-induced cytokine production in various strains of mice

The exogenous addition of GM-CSF to the splenocyte culture system enhanced cytokine induction by SCG in C57BL/6 mice. We, therefore, examined the splenocytes from various strains of mice. Splenocytes from C3H/HeN, C3H/HeJ, BALB/c, C57BL/6, and DBA/2 mice were cultured with SCG (100 μg/ml) in the presence of rMuGM-CSF (1 ng/ml) *in vitro* to collect the supernatant. The induction of IFN-γ was not detected or was at an insignificant level after SCG treatment in almost all strains of mice except for DBA/2 mice (Fig. 7). By exogenously adding rMuGM-CSF to the splenocyte culture system, the concentration of IFN-γ significantly increased due to SCG in splenocytes from all strains. These results suggested that exogenous or endogenous GM-CSF (or both) is a key reaction required by splenocytes to respond to SCG-induced cytokines.

Next, to determine whether the GM-CSF expression characteristic in DBA/2 mice is a dominant or recessive trait, we examined the splenocytes from C57BL/6 × DBA/2 F_1 hybrid (BDF1-hybrid) mice. Splenocytes from C57BL/6, BDF1, or DBA/2 mice were cultured with SCG (100 μg/ml) in the presence of rMuGM-CSF (1 ng/ml) *in vitro* to collect the supernatant. The induction of IFN-γ was not detected or was at an insignificant level after SCG treatment in C57BL/6 and BDF1 mice (Fig. 8). By exogenously adding rMuGM-CSF to the splenocyte culture system, the concentration of IFN-γ significantly increased due to SCG in splenocytes from all strains. These

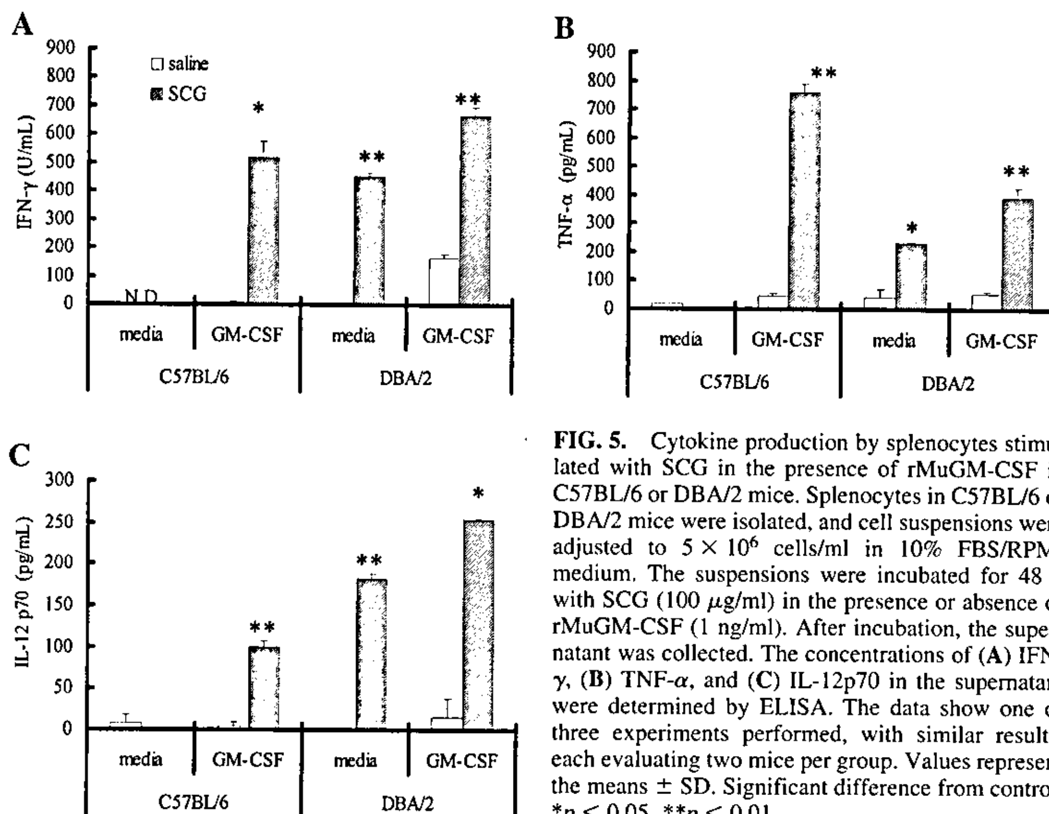

FIG. 5. Cytokine production by splenocytes stimulated with SCG in the presence of rMuGM-CSF in C57BL/6 or DBA/2 mice. Splenocytes in C57BL/6 or DBA/2 mice were isolated, and cell suspensions were adjusted to 5×10^6 cells/ml in 10% FBS/RPMI medium. The suspensions were incubated for 48 h with SCG (100 μg/ml) in the presence or absence of rMuGM-CSF (1 ng/ml). After incubation, the supernatant was collected. The concentrations of **(A)** IFN-γ, **(B)** TNF-α, and **(C)** IL-12p70 in the supernatant were determined by ELISA. The data show one of three experiments performed, with similar results, each evaluating two mice per group. Values represent the means ± SD. Significant difference from control, $*p < 0.05$, $**p < 0.01$.

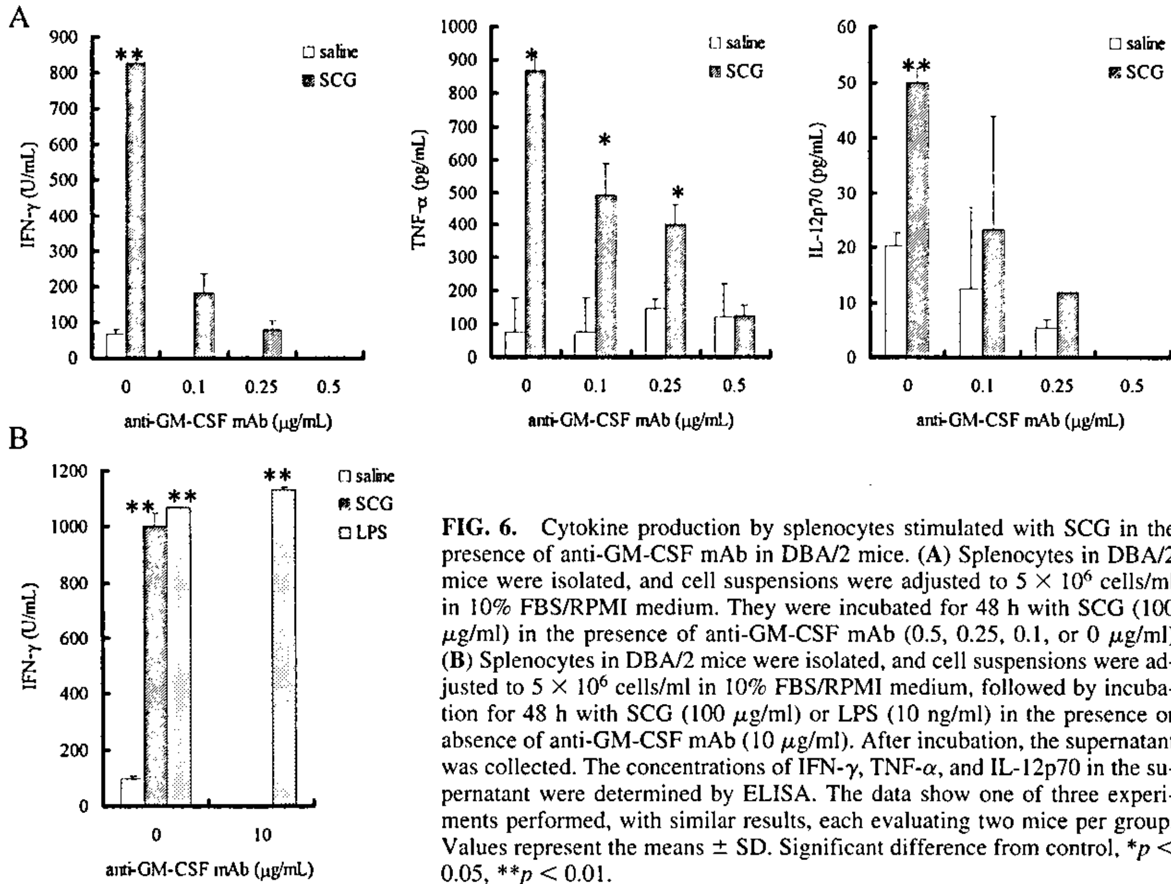

FIG. 6. Cytokine production by splenocytes stimulated with SCG in the presence of anti-GM-CSF mAb in DBA/2 mice. (**A**) Splenocytes in DBA/2 mice were isolated, and cell suspensions were adjusted to 5×10^6 cells/ml in 10% FBS/RPMI medium. They were incubated for 48 h with SCG (100 μg/ml) in the presence of anti-GM-CSF mAb (0.5, 0.25, 0.1, or 0 μg/ml) (**B**) Splenocytes in DBA/2 mice were isolated, and cell suspensions were adjusted to 5×10^6 cells/ml in 10% FBS/RPMI medium, followed by incubation for 48 h with SCG (100 μg/ml) or LPS (10 ng/ml) in the presence or absence of anti-GM-CSF mAb (10 μg/ml). After incubation, the supernatant was collected. The concentrations of IFN-γ, TNF-α, and IL-12p70 in the supernatant were determined by ELISA. The data show one of three experiments performed, with similar results, each evaluating two mice per group. Values represent the means ± SD. Significant difference from control, $*p < 0.05$, $**p < 0.01$.

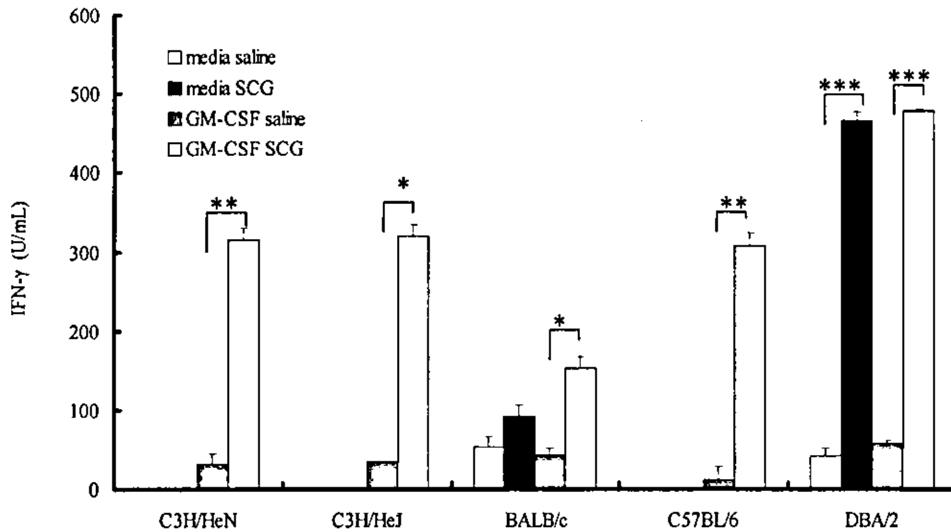

FIG. 7. IFN-γ production by splenocytes stimulated with SCG in the presence of rMuGM-CSF in various strains of mice. Splenocytes in C3H/HeN, C3H/HeJ, BALB/c, C57BL/6, and DBA/2 mice were isolated, and cell suspensions were adjusted to 5×10^6 cells/ml in 10% FBS/RPMI medium. After incubation for 48 h with SCG (100 μg/ml) in the presence or absence of rMuGM-CSF (1 ng/ml), the supernatant was collected, and the concentration of IFN-γ in the supernatant was determined by ELISA. The data show one of three experiments performed, with similar results, each evaluating two mice per group. Values represent the means ± SD. Significant difference from control, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

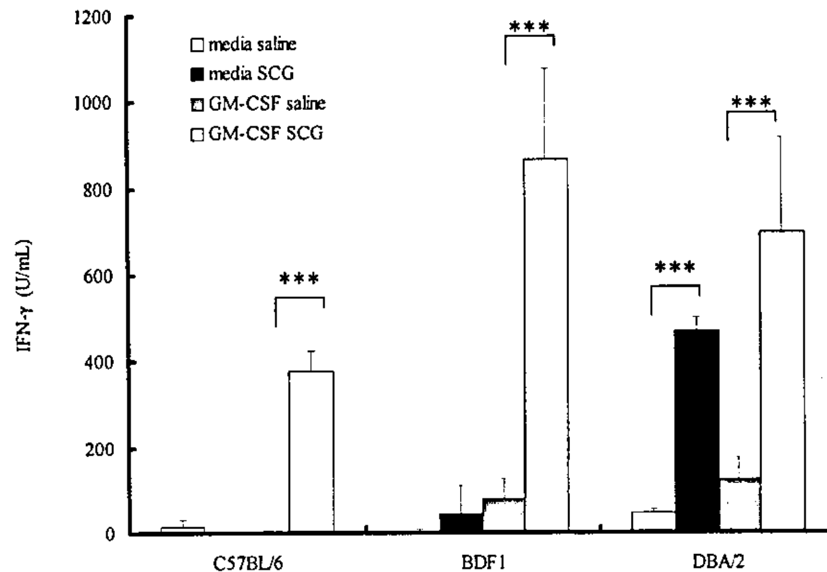

FIG. 8. IFN-γ production by splenocytes stimulated with SCG in the presence of rMuGM-CSF in F_1 mice. Splenocytes in C57BL/6, BDF1, and DBA/2 mice were isolated, and cell suspensions were adjusted to 5×10^6 cells/ml in 10% FBS/RPMI medium. After incubation for 48 h with SCG (100 μg/ml) in the presence or absence of rMuGM-CSF (1 ng/ml), the supernatant was collected, and the concentration of IFN-γ in the supernatant was determined by ELISA. The data show one of two experiments performed, with similar results, each evaluating three mice per group. Values represent the means ± SD. Significant difference from control, $***p < 0.001$.

facts strongly suggest that the susceptibility to SCG is conferred as a recessive genetic trait present in DBA/2 mice.

Effect of SCG on T cells or other nonadherent splenocytes in DBA/2 mice

We previously reported that DBA/2 mice contain significantly higher titers of antibody to SCG than other inbred naive mice.[13] To examine if antibody to SCG had an effect on IFN-γ induction by SCG in DBA/2 mice, B cell-depleted splenocytes were prepared as follows and tested for IFN-γ productivity. The splenocytes derived from DBA/2 mice were cultured *in vitro* for 1.5 h in plastic dishes. B cells were depleted from nonadherent splenocytes, and T cells were enriched by being passed through passing nylon wool columns. The whole nonadherent cells or the CPF was mixed with adherent cells, and these cells were cultured in the presence or absence of SCG (100 μg/ml) *in vitro*. As shown in Figure 9, IFN-γ and GM-CSF were increased by SCG in mixtures of adherent cells and CPF as well as nonadherent cells. That B cell depletion from splenocytes had no effect on the induction of IFN-γ and GM-CSF by SCG suggested that B cells and their products may not be involved in the mechanism of IFN-γ induction by SCG. These results also suggested that T cells may play an important role in IFN-γ induction by SCG. As shown in Figure 9B, GM-CSF and IFN-γ were increased by SCG in adherent cells but not in nonadherent cells or CPF. However, even in adherent cells, the level of GM-CSF release was lower than that in coexistent adherent cells and nonadherent cells or CPF.

The finding that GM-CSF production was significantly increased under the coexistence of adherent cells and T cell enriched fraction suggested that cell—cell contact was required for induction of GM-CSF by SCG to induce IFN-γ.

DISCUSSION

We have been working on the relation between structure and immunomodulating activity of β-glucans using *Grifola frondosa*[11,14] *Peziza vesiculosa*,[15] *Sclerotinia sclerotiorum*,[16,17] *Schizophyllum commune*,[8] *Omphalia lapidescens*,[18] *Ganederma lucidum*,[19] *Saccharomyces cerevisiae*,[20] *Candida albicans*,[21] *S. crispa*, [3–7,12,13] and *Malassezia furfur*.[22] We found that in a preclinical animal study that activity was significantly dependent on molecular weight, degree of branching, and conformation.[23,24] Cancer immunotherapy used for many patients and enhancement of the quality of life of patients are important goals of cancer treatment. The establishment of a molecular mechanism for use in biotherapy is still needed. In a previous study, we reported that a gel-forming 6-branched 1,3-β-glucan induced macrophages to produce several mediators, including the inflammatory cytokines IL-1, IL-6, TNF-α, and NO.[8–11] The activities of β-glucan on other leukocytes and functional β-glucan receptors on such leukocytes are not clear. Cytokine induction by β-glucan from leukocytes derived from normal mice was low. We recently showed that naive DBA/1 and DBA/2 mice, but not other strains of mice, produced significant amounts of IFN-γ due to SCG.[12] In the present study, SCG stimulated splenocytes from DBA/2 mice to produce such cytokines as IFN-γ, TNF-α GM-CSF, and IL-12p70 (Fig. 1). GM-CSF is a key cytokine among them. In fact, neutralizing

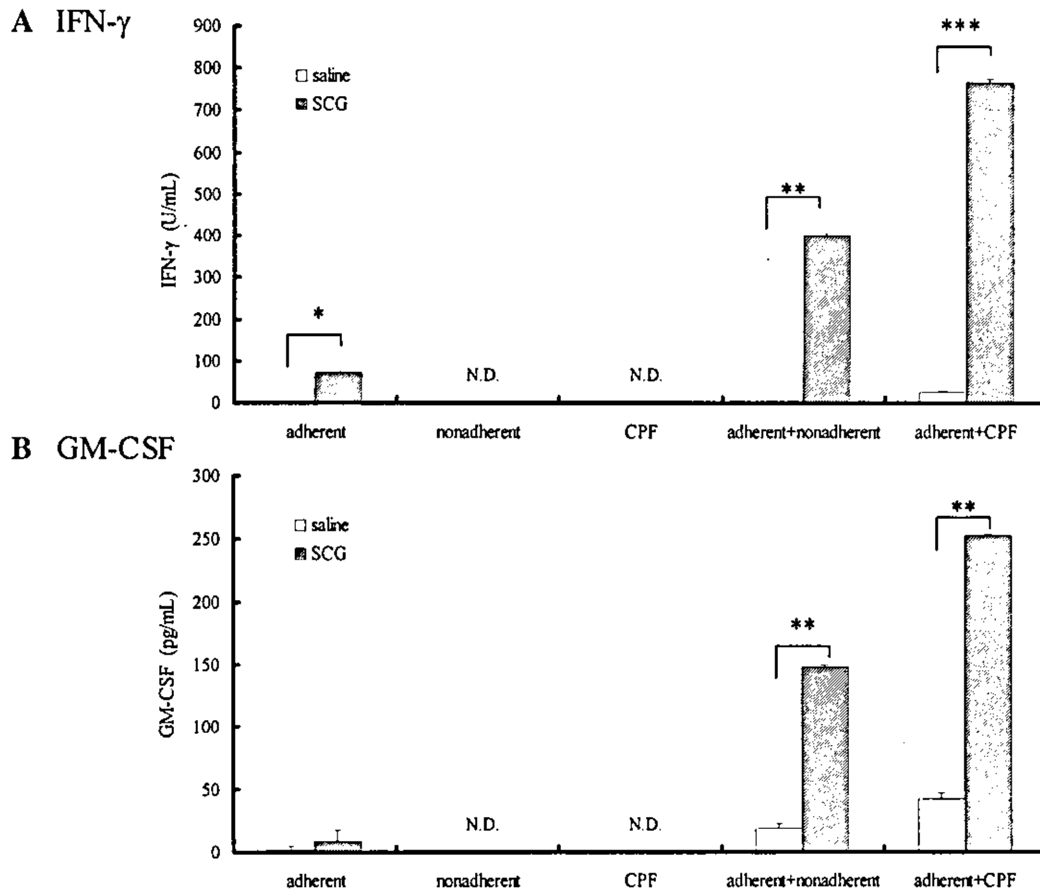

FIG. 9. Cytokine production by splenocytes, mixed adherent, and nonadherent T cells derived from DBA/2 mice. Splenocytes in DBA/2 mice were isolated, and adherent cells, nonadherent cells, or CPF was prepared. These cells were reconstructed by mixing. Cell suspensions were incubated for 48 h in the presence or absence of SCG (100 μg/ml). After incubation, the supernatant was collected. The IFN-γ (**A**) and GM-CSF (**B**) concentrations in the supernatant were determined by ELISA. The data show one of four experiments performed, with similar results. Values represent means ± SD. Significant difference from control, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

GM-CSF using anti-GM-CSF mAb significantly inhibited IFN-γ, TNF-α, and IL-12p70 production elicited by SCG (Fig. 6).

The fact that splenocytes from DBA/1 and DBA/2 mice reacted strongly with SCG suggested that these cells are sensitive to SCG *in vitro*. However, studies on the reason for the high sensitivity to SCG in DBA/2 and DBA/1 cells have been superficial. In this study, the exogenous addition of GM-CSF to the splenocyte culture system in various strains of mice synergistically enhanced IFN-γ, TNF-α, IL-12p70, and GM-CSF induction by SCG (Figs. 2E, 3, 4, and 7). Therefore, cells reacted with GM-CSF were sensitive to SCG *in vitro* to produce cytokines. In contrast, in DBA/2 mice, cells were already activated (Fig. 5). These results suggested that GM-CSF is a key molecule for cytokine induction by β-glucan, and GM-CSF induction by SCG is the specific step in DBA/2 mice *in vitro*.

We previously reported that DBA/2 mice have significantly higher titers of antibody to SCG than other inbred naive mice.[13] As shown in Figure 9, IFN-γ and GM-CSF were increased by SCG in mixtures of adherent splenocytes and B cell-depleted fractions as well as nonadherent fractions. That B cell

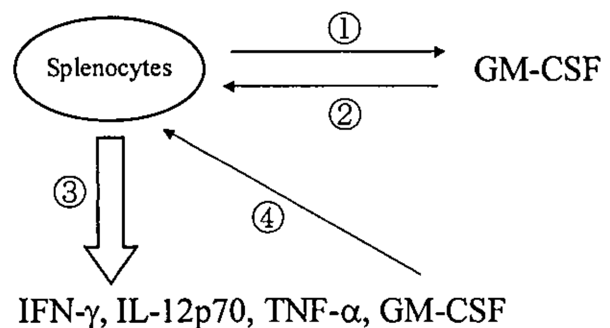

FIG. 10. Model for the mechanism of cytokine induction by SCG in DBA/2 mice. In DBA/2 mice, (**1**) SCG activates the splenocytes to produce GM-CSF, as a first signal. The resulting splenocytes (**2**) are again stimulated by SCG to produce additional cytokines such as IFN-γ, TNF-α, IL12p70, and GM-CSF (**3**). The increased concentration of GM-CSF (**4**) further activates splenocytes to augment whole activation cascades.

depletion from splenocytes did not affect the induction of IFN-γ and GM-CSF by SCG suggested that B cells and the specific antibody may not be involved in the mechanism of cytokine induction by SCG. These results also suggested that T cells may play an important role in IFN-γ induction by SCG. We also reported previously that IFN-γ was induced by SCG in adherent splenocytes, but IFN-γ production was most significantly increased by SCG in instances involving the coexistence of adherent and nonadherent splenocytes in DBA/2 mice.[12] As shown in Figure 9, in DBA/2 mice, GM-CSF and IFN-γ were increased by SCG in adherent cells, but the level of GM-CSF release was lower than that in coexistent adherent cells and nonadherent or T cell-enriched fraction. The finding that GM-CSF production was significantly increased under the coexistence of adherent and nonadherent cells or a T cell-enriched fraction suggests that cell-cell contact was required for induction of GM-CSF by SCG to induce IFN-γ. These results suggest that in DBA/2 mice, SCG activate the splenocytes to produce GM-CSF, as a first signal. The resulting splenocytes are again stimulated by SCG to produce additional cytokines such as IFN-γ, TNF-α, IL-12p70, and GM-CSF. The increased concentration of GM-CSF further activates splenocytes to augment whole activation cascade. These results suggest that GM-CSF induction by SCG is a key step and the specific step in DBA/2 mice *in vitro* (Fig. 10).

Murine leukocytes are activated by endotoxin/bacterial LPS and produce various cytokines and chemical mediators, such as TNF-α, IL-1, IL-6, IL-8, NO, and IFN-γ. To clarify whether this response to splenocytes by SCG was an SCG-specific reaction, we compared the ability of SCG and LPS to induce cytokines in the presence of rMuGM-CSF. Cytokine production induced by SCG in the presence of GM-CSF was higher than that induced by LPS in C57BL/6 mice (Fig. 2B). Additionally, neutralizing GM-CSF using anti-GM-CSF mAb significantly inhibited cytokines elicited by SCG in DBA/2 mice but had no influence on cytokine induction by LPS (Fig. 6). Therefore, splenocytes modulated by GM-CSF specifically responded to SCG to produce cytokines, and cytokine induction was not mediated by contaminating LPS.

GM-CSF is a 23-kDa glycoprotein known as a hematopoietic growth factor required for the proliferation and survival of hematopoietic cells committed to granulocytic and macrophage cell lineages and myeloid leukemic cells.[25–27] It is also required for the differentiation of these cells into neutrophilic or eosinophilic granulocytes, macrophages, bone marrow macrophages, or dendritic cells (DCs).[27–31] GM-CSF increases the responsiveness of tissue macrophages to M-CSF.[25] In addition to stimulating the production of granulocytes, macrophages, and DCs, GM-CSF has a pronounced capacity to increase the function of these cells in a variety of immune reactions.[32] Its *in vivo* effects include rapid leukocytosis,[32] increased numbers of granulocytes and macrophages in tissues,[32] stimulation of phagocytosis and superoxide production by neutrophils and macrophages,[33] induction of class II major histocompatibility complex (MHC) expression and urokinase-type plasminogen activator production by monocyte/macrophages,[34] enhancement of granulocyte and monocyte cell adhesion,[35,36] augmentation of antigen-presenting function in macrophages,[37] enhancement of production of cytokines by mononuclear cells,[38] and enhancement of chemotaxis of neutrophils, monocytes, or DCs by transendothelial migrating monocytes.[39–41] Thus, GM-CSF has the ability to modulate adherent cell functions. Previously, it was suggested that adherent cells in splenocytes derived from DBA/2 mice were β-glucan responsive.[12] Our results suggest that endogenously produced GM-CSF modulates adherent cells, and the population of cells responsible for SCG increases in DBA/2 mice.

The facts that in splenocytes from DBA/2 mice, high amounts of cytokines are induced by SCG *in vitro* and DBA/2 mice contain significantly higher titers of antibody to SCG than other inbred naive mice[13] suggest that DBA/2 mice are highly sensitive to SCG in a hereditary manner. In this study, the induction of IFN-γ was not detected or was at an insignificant level after SCG treatment, and by exogenously adding rMuGM-CSF to the splenocyte culture system, the concentration of IFN-γ significantly increased due to SCG in C57BL/6 $\times$ DBA/2 F_1-hybrid (BDF1-hybrid) mice (Fig. 8). Mice from the F_1 cross exhibited little to no IFN-γ production, indicating that the susceptibility to SCG is conferred as a recessive genetic trait. Analysis of the genes controlling the susceptibility to β-glucan-induced IFN-γ production could lead to the development of clinically applicable effective immunotherapy using β-glucan.

The complement receptor type 3 (CR3) is a glucan receptor of phagocytes.[42] Recently, dectin-1[43] and toll-like receptors (TLRs)[44,45] were reported to be candidates for new glucan receptors. The induction of cytokines in the presence of GM-CSF by SCG may lead to the discovery of new glucan receptors or allow the description of preexisting receptors. Willment et al.[46] reported that dectin-1 expression and function are enhanced in GM-CSF-treated macrophages. Therefore, receptors, such as dectin-1, should be highly expressed on adherent splenocytes in DBA/2 mice because of endogenously produced GM-CSF, and splenocytes in DBA/2 mice should be highly responsive to SCG-induced cytokine production *in vitro*.

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Protective Properties of Neoechinulin A against SIN-1–Induced Neuronal Cell Death

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Peroxynitrite ($ONOO^-$) is thought to be involved in the neurodegenerative process. To screen for neuroprotective compounds against $ONOO^-$-induced cell death, we developed 96-well based assay procedures for measuring surviving cell numbers under oxidative stress caused by 3-(4-morpholinyl) sydnonimine hydrochloride (SIN-1), a generator of $ONOO^-$, and sodium *N,N*-dietyldithiocarbamate trihydrate (DDC), an inhibitor of Cu/Zn superoxide (O_2^-) dismutase. Using these procedures, we obtained a microbial metabolite that rescued primary neuronal cells from SIN-1-induced damage, but not from DDC-induced damage. By NMR analysis, the compound was identified as neoechinulin A, an antioxidant compound that suppresses lipid oxidation. We found that the compound rescues neuronal cells such as primary neuronal cells and differentiated PC12 cells from damage induced by extracellular $ONOO^-$. However, non-neuronal cells, undifferentiated PC12 cells and cells of the fibroblast cell line 3Y1 were not rescued. Neoechinulin A has scavenging, neurotrophic factor–like and anti-apoptotic activities. This compound specifically scavenges $ONOO^-$, but not O_2^- or nitric oxide (NO). Similar to known neuroprotective substances such as nerve growth factor and extracts of *Gingko biloba* leaves, neoechinulin A inhibits the SIN-1-induced activation of caspase-3–like proteases and increases NADH-dehydrogenase activity. These results suggest that neoechinulin A might be useful for protecting against neuronal cell death in neurodegenerative diseases.

Key words: free radical scavengers, neoechinulin A, neuroprotective effect, oxidative stress, peroxynitrite.

Peroxynitrite ($ONOO^-$) is produced from superoxide (O_2^-) and nitric oxide (NO) (*1*). O_2^- is highly toxic to neurons as it initiates the chain-reactive production of various reactive oxygen species (ROS) during metabolism; protection against O_2^--induced toxicity is critical for neuronal survival (*2, 3*). NO has diverse physiological functions (*4–7*) and is toxic to neuronal cells (*8*). NO reacts with O_2^- in a diffusion-limited manner to form the more toxic oxidant $ONOO^-$ (*1*), which induces the death of PC12 cells (*9–11*) and cortical neurons (*12*). In the central nervous system, $ONOO^-$ can be generated by microglial cells activated by pro-inflammatory cytokines or β-amyloid peptide and by neurons (*13*). $ONOO^-$ is far more selective than other strong oxidant and preferentially reacts with thiols (*14*). In addition, $ONOO^-$ also reacts with tyrosine to yield 3-nitrotyrosine (*15*). Increasing levels of nitrotyrosine (*16*) are associated with degenerating neurons in the Alzheimer's disease brain, suggesting pathogenic roles for $ONOO^-$.

SIN-1 (3-(4-morpholinyl) sydnonimine hydrochloride) is a vasodilator that spontaneously releases O_2^- and NO into the medium, thereby producing $ONOO^-$ (*17, 18*). The compound causes a concentration-dependent increase in cortical cell injury (*19*). It has been reported that neurotrophic factors such as nerve growth factor (NGF) (*20*), and free radical scavengers such as uric acid (*21*) and manganese (III) tetrakis (4-carboxyphenyl) porphyrin (Mn-TBAP) (*22*), rescue neuronal cells from SIN-1-induced damage. However, these compounds prevent oxidative damage caused by various ROS as well as $ONOO^-$ -induced damage.

Copper/zinc superoxide dismutase (Cu/Zn-SOD) is highly expressed in neurons (*23*). Thus, an SOD-inhibitor, sodium *N,N*-dietyldithiocarbamate trihydrate (DDC) elevates the amounts of intracellular O_2^- and induces oxidative damage through the chelathor of Cu^{2+} in the active site of Cu/Zn-SOD (*24, 25*). To obtain compounds that specifically protect neuronal cells against $ONOO^-$-induced oxidative damage, we screened microbial metabolites that rescue primary neuronal cells from SIN-1-induced injury, but not from DDC-induced injury. We obtained a microbial metabolite that specifically protects against $ONOO^-$-induced cell death. In this paper, we describe the neuroprotective properties of this compound.

MATERIALS AND METHODS

Culture of Fungi and Extraction of Their Metabolite—Fungi were isolated as described by Inoue *et al.* (*26*) and incubated at room temperature for 21 d. Each culture was filtered through cheesecloth to remove the mycelia, and the components were extracted with CH_2Cl_2. The

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organic extract was evaporated *in vacuo* to yield a crude extract, which was subjected repeatedly to silica gel column chromatography using *n*-hexane–ethyl acetate as the solvent.

Cell Culture and 96-Well Based Assay Procedures—Primary neuronal cells were prepared from embryonic 17-d Wister rat brains as described by Suzumura *et al.* (*27*). In brief, the meninges were removed and the brains were dissociated by adding in Dulbecco's modified Eagle's medium (DMEM) MIXTURE F-12 HAM (Sigma Aldrich Fine Chemicals, St. Louis, MO, USA) containing 2.85 mg/ml glucose, 5 μM HEPES, 25 μg/ml insulin, 2 μM progesterone, 0.1 mM putrescine, 0.03 μM sodium selenite, 0.1 mg/ml apo-transferrin, 100 U/ml penicillin and 100 μg/ml streptomycin (DF medium).

For screening microbial metabolites, primary neuronal cells were cultured on poly-D-lysine (PDL) coated 96-well plastic plates (Becton Dickinson, NJ, USA) at an initial density of 0.7×10^5 cells/cm^2 in 2% fetal bovine serum (FBS)–DF medium for 5 d at 37°C. The cultures were treated with microbial metabolites for 24 h, and then, cell death was induced by adding 1 mM SIN-1 (Dojindo, Kumamoto Japan) or 4 μg/ml DDC (Wako, Osaka, Japan). After 24 h, and live cells were counted using a Cell Counting Kit-8 (Dojindo). The kit detects mitochondrial NADH-dehydrogenase activity in live cells by measuring the reduction of the tetrazolium monosodium salt, WST-8. This is a modified MTT assay, and it is known that the MTT assay is not influenced in the presence of various oxidants. Cell number was also measured by the CyQUANT Cell Proliferation Assay Kit (Molecular Probes, Eugene, USA), which quantifies the amount of DNA (*28*). The chemical structures of the compounds obtained were determined by NMR analysis.

PC12 cells were incubated in 75-cm^2 tissue culture flasks in DMEM (Nissui, Tokyo, Japan) supplemented with 10% FBS, 5% horse serum (HS), 100 U/ml penicillin, and 100 μg/ml streptomycin. NGF-differentiated PC12 cells were treated with 100 ng/ml NGF for 5 d. Cells of the rat fibroblast cell line 3Y1 were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

To study the protective effects of microbial metabolites and known scavengers on PC12 cells and 3Y1 cells, cells were cultured at a initial density of 3.0×10^3 cells/cm^2 in DMEM supplemented with 10% FBS, 5% HS or DMEM containing 10% FBS 5 d at 37°C. Cell death induced by SIN-1 and cell viability were measured as described above.

All cultures were maintained at 37°C in a humidified CO_2-incubator.

Observation of Tyrosine Nitration by Immunofluorescence—Primary neuronal cells were cultured for 7 d on PDL-coated 8-well culture slides (Becton Dickinson). The cells were fixed with 4% paraformaldehyde in PBS (+) (phosphate-buffered saline containing 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+}) for 1 h, washed three times with PBS (+), and incubated with 10% FBS-PBS (+) at 4°C for 1 h. Next, they were incubated with an anti-nitrotyrosine antibody 1AE (Upstate, VA, USA) in 10% FBS-PBS (+) at 4°C for 1 h. After two washes with PBS (+), the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (H&L) (ICN Pharmaceuticals, Inc. Morgan, Irvine, CA, USA) in 10% FBS-PBS (+) for 1 h. Cell nuclei were stained with 10 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI) (Sigma Aldrich Fine Chemicals) at 37°C for 30 min. The cells were then washed with PBS (+), and the nuclear DNA was observed under a fluorescence microscope IX70 (OLYMPUS, Tokyo, Japan).

Measurement of Scavenging Activity—We measured $ONOO^-$ and O_2^- scavenging activities using the chemiluminescent procedure described by Radi *et al.* (*29*) and Beauchamp and Fridovich (*30*), respectively. In brief, 400 mM 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) (Sigma Aldrich Fine Chemicals) was diluted with 5 mM $NaHCO_3$, pH 10.5 (Wako) in Hank's balanced salt solution (HBSS) (Invitrogen Corp, Carlsbad, CA, USA) (solution A). Test compounds were diluted with HBSS (pH 7.0) (solution B). Solutions A and B and 0.5 mM SIN-1 diluted with HBSS (pH 7) were mixed, and then $ONOO^-$ was measured using a chemiluminometer (MicroLumat LB96V; Berthold Technology, Bad Wildbad, Germany) for 20 min. O_2^- was generated by the xanthine-xanthine oxidase system following the modified method of Beauchamp and Fridovich (*30*). Specimens in Tris-HCl buffer were added to the wells of 96-well plates, each well containing 10 U/ml xanthine oxidase and 2 μM 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydriimidazo-[1,2-α]pyrazin-3-one (MCLA; Tokyo Kasei Co. Ltd, Tokyo, Japan). To generate O_2^-, 30 μM xanthine was added to each well. Chemiluminescence intensity was measured with a chemiluminometer for 10 min. NO scavenging activity was measured by the fluorometric method of Kojima *et al.* (*31*). The fluorometric detection of NO was carried out using the NO indicator (NONOate; Dojindo) and diaminofluorescein-2 (DAF-2; Daiichi Pure Chemicals Co. Ltd, Tokyo, Japan). The fluorescence from DAF-2T, the reaction product of DAF-2 with NO, was measured as the fluorescence intensity of DAF-2T using a microplate fluorescence reader (Packard Instrument Co., Meriden, USA) (Ex = 490 nm and Em = 520 nm). Mn-TBAP (Dojindo), a scavenger of $ONOO^-$ and O_2^-, and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (C-PTIO, Dojindo), a scavenger for NO, were used as controls.

Caspase Activity Assay—We measured caspase-3 activity according to Zhou *et al.* (*32*). After exposure to $ONOO^-$, cells were incubated in 50 mM Tris-HCl buffer (pH7.4) containing 4 mM DTT (Sigma Aldrich Fine Chemicals), 2 mM EDTA, 10% glycerol, 0.1% Triton X-100 and 20 μM Ac-Asp-Glu-Val-Asp-7-amido-4-methylcoumarine (Sigma Aldrich Fine Chemicals), a fluorogenic substrate for caspase-3. The activity of caspase-3-like proteases was determined by measuring the fluorescence intensity of the cleaved substrate in a microplate fluorescence reader (Packard Instrument Co., Meriden, USA) (Ex = 360 nm and Em = 460 nm).

RESULTS

Screening of Microbial Metabolites That Prevent SIN-1–Induced Neuronal Cell Death—We screened two hundred microbial metabolites and obtained one compound that rescued primary neuronal cells from SIN-1-induced death. This compound was identified as neoechinulin A by NMR analysis. In the presence of SIN-1, the viability

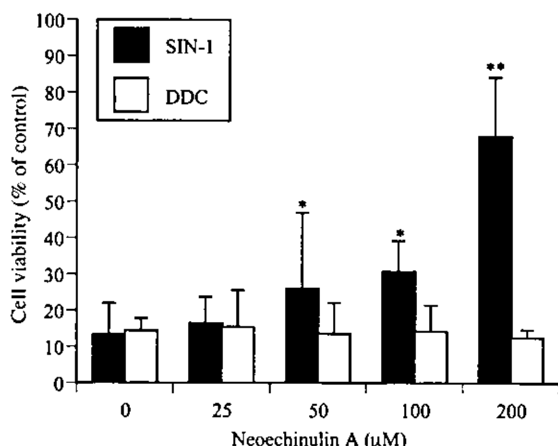


Fig. 1. **Dose-dependent effects of neoechinulin A on neuronal cells.** Primary neuronal cells were seeded at a density of 1.4 × 10^5 cells/cm^2. After 5 d, cells were incubated with neoechinulin A for 24 h, and treated with 1 mM SIN-1 or 4 μg/ml DDC for 24 h. Cell viability was measured using a Cell Counting Kit-8 (means ± SD, n = 4). $^*p < 0.05$, $^{**}p < 0.01$.

of primary neuronal cells was less than 15%. Treatment with 200 μM neoechinulin A increased cell viability to more than 60%, while such treatment provided no protection against cell death induced by DDC (Fig. 1). This indicates that neoechinulin A protects primary neuronal cells against $ONOO^-$-induced death, but not against O_2^--induced death.

3Y1 cells, a fibroblast cell line, were not rescued from SIN-1–induced death by neoechinulin A. To determine the protective specificity of neoechinulin A, its effect on NGF-differentiated and undifferentiated PC12 cells was examined. Neoechinulin A rescued only differentiated PC12 cells (Fig. 2), suggesting that protective properties of the compound are specific to neuronal cells. Differentiated PC12 cells were also dose-dependently rescued by neoechinulin A (Fig. 3.). The results, shown in the Figures 2 and 3, were confirmed using another cell counting kit, the CYQUANT Cell proliferation kit (data not shown).

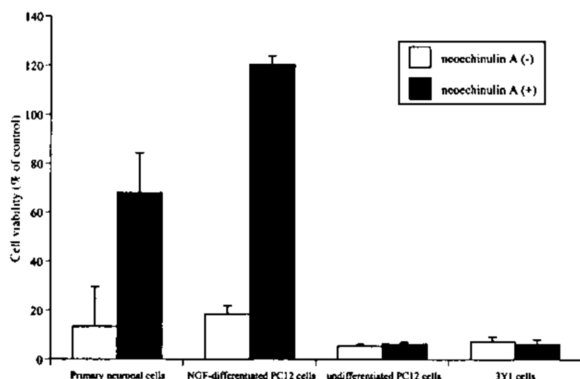


Fig. 2. **Preventive effect of neoechinulin A against SIN-1 injury.** Primary neuronal cells (1.4 × 10^5 cells/cm^2), PC12 cells (3.0 × 10^3 cells/cm^2) and 3Y1 cells (3.0 × 10^3 cells/cm^2) were cultured on PDL-coated 96-well plates for 5 d. After treatment with or without 200 μM neoechinulin A for 24 h, cells were cultured in the presence of 1 mM SIN-1 for 24 h. Cell viability was determined using a Cell Counting Kit-8.

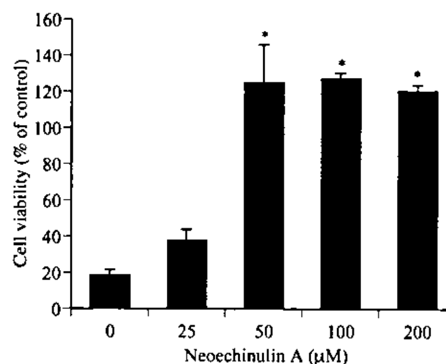


Fig. 3. **Dose-dependent effects of neoechinulin A on differentiated PC12 cells.** PC12 cells were seeded at a density of 3.0 × 10^3 cells/cm^2 and treated with 100 ng/ml NGF for days. Differentiated PC12 cells were incubated with neoechinulin A for 24 h, and treated with 1 mM SIN-1 for 24 h. Cell viability was measured using a Cell Counting Kit-8. (means ± SD, n = 4) $^*p < 0.01$.

Ability of Neoechinulin A to Scavenge ROS Produced by SIN-1—To study the scavenging activity of neoechinulin A, we examined the nitration of tyrosine residues in SIN-1–exposed cells by immunostaining with monoclonal anti-nitrotyrosine antibody 1AE. Tyrosine nitration was almost completely inhibited by neoechinulin A (Fig. 4), suggesting that neoechinulin A has $ONOO^-$ scavenging activity. This activity was confirmed by the chemiluminescence procedure. The $ONOO^-$ scavenging activity of neoechinulin A is comparable to the activities of Mn-TBAP and C-PTIO (Fig. 5A). Scavenging either O_2^- or NO also prevents the generation of $ONOO^-$ from SIN-1. Mn-TBAP and SOD scavenge about 100% and 75%, respectively of O_2^-, while neoechinulin A does not scavenge O_2^- (Fig. 5B). The fluorometric procedure using DAF-2 revealed that C-PTIO scavenges about 60% of NO, while neoechinulin A does not eliminate NO (Table 1). These results indicate that neoechinulin A specifically scavenges $ONOO^-$, but not O_2^- and NO.

Neuroprotective Effects of Neoechinulin A in SIN-1-Induced Oxidative Stress—It has been reported that various substances, such as anti-apoptotic compounds and neurotrophic factors, have neuroprotective activities (*33*, *34*). We confirmed the neuroprotective effect of the scavengers, C-PTIO and uric acid (Fig. 6A). To examine the activities of neoechinulin A other than scavenging activity, differentiated PC12 cells were pretreated with neoechinulin A, C-PTIO and uric acid for 24 h, and then, after removal of the compounds, with SIN-1 for 24 h. Under these conditions, the scavengers did not rescue differentiated PC12 cells. As shown in Fig. 6B, neoechinulin A still had a neuroprotective effect. This result suggests that neoechinulin A activities other than its scavenging activity.

Figure 3 shows that the viability of neoechinulin A–treated cells was increased by 120%. In this study, live cells were counted with a cell counting kit that measures NADH-dehydrogenase activity as described in "MATERIALS AND METHODS." Several researchers have reported that NGF and bFGF increase the mitochondrial NADH-

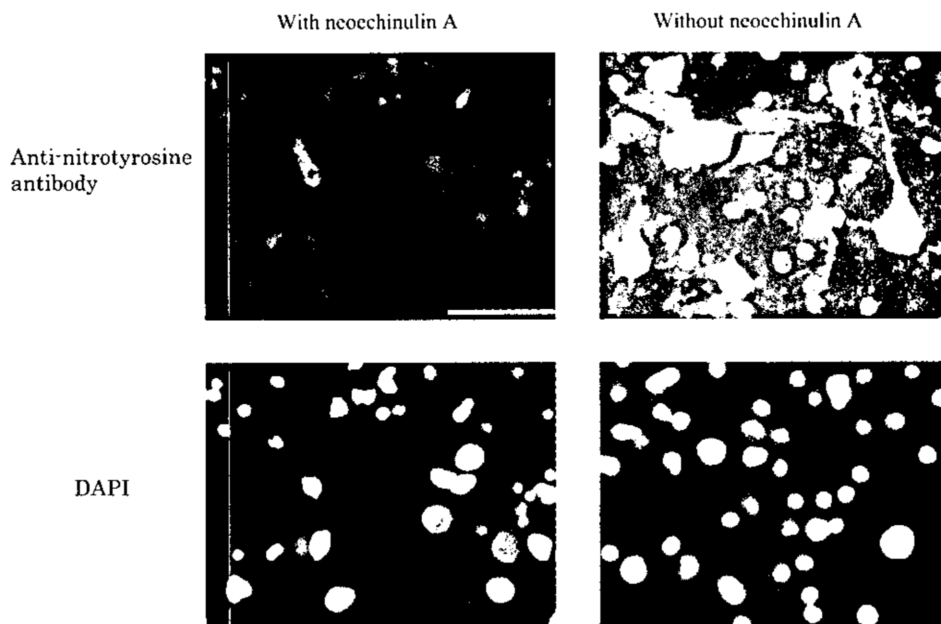


Fig. 4. **Prevention of ONOO⁻-induced tyrosine nitration by neoechinulin A.** Primary neuronal cells were incubated with 0.25 mM SIN-1 for 2 h with or without 200 μM neoechinulin A, and stained with an anti-nitrotyrosine antibody 1AE and DAPI as described in "MATERIALS AND METHODS." Bar = 50 μm

dehydrogenase activity of PC12 cells (*35, 36*). These facts suggest that neoechinulin A activates this enzyme in PC12 cells. To examine this possibility, we investigated the effect of neoechinulin A on NADH-dehydrogenase in differentiated PC12 cells in the absence of SIN-1. NADH-dehydrogenase activity was normalized to the number of cells as measured with the assay kit that counts cell number based on quantification of genomic DNA. A significant increase in NADH-dehydrogenase activity was observed after the addition of neoechinulin A (Fig. 7).

$ONOO^-$ activates caspase-3 (*37, 38*). Thus, we examined the effect of neoechinulin A on the SIN-1–induced activation of caspase-3-like proteases in differentiated PC12 cells. The activation was suppressed by about 65% by 200 μM neoechinulin A. On the other hand, neoechinulin A did not influence the caspase-3–ike protease activity of 3Y1 cells (Fig. 8). These results indicate that neoechinulin A has neurotrophic factor-like and anti-apoptotic activities.

DISCUSSION

Neoechinulin A is a known metabolite of *A. rubber* and *A. amstelodami* (*26*). Yagi *et al.* (*39*) reported neoechinulin A to be an antioxidant compound that suppresses lipid peroxidation in dried bonito flakes, *Katsuobushi*. However, additional properties of the compound were not investigated. In this paper, we show that neoechinulin A has scavenging, neurotrophic factor-like and anti-apoptotic activities. The results shown in Fig. 5 and Table 1 indicate that the compond scavenges only $ONOO^-$ released

Table 1. **NO generation in the presence of scavengers.**

Compounds	Generation of NO (% of control)
None	100
Neoechinulin A	100 ± 3
C-PTIO	41 ± 1
SOD	108 ± 5

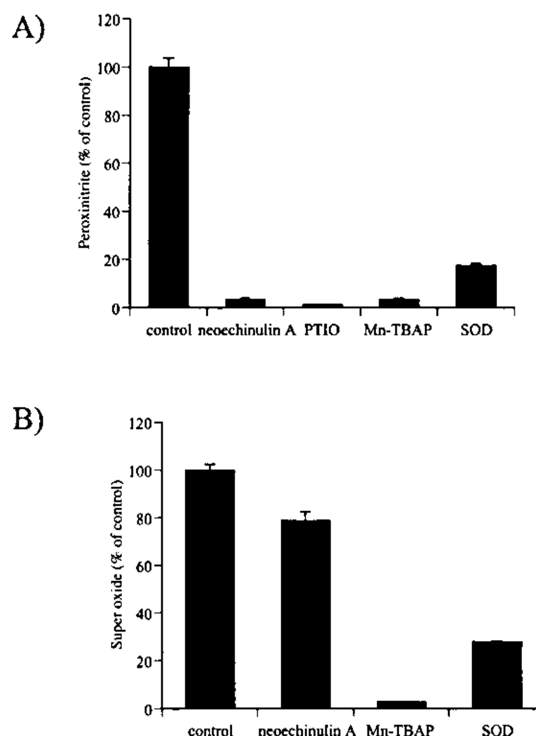
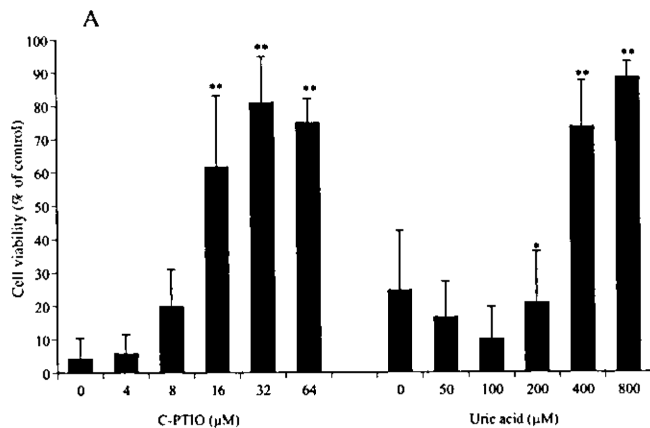


Fig. 5. **Scavenging activity of neoechinulin A.** (A) Measurement of $ONOO^-$ by luminol-dependent chemiluminescence. Luminol was diluted to 400 mM with 5 mM $NaHCO_3$ (pH 10.5) and the samples were diluted with HBSS, pH 7, prior to the measurement of chemiluminescence. Just before measurement, 0.5 mM SIN-1 diluted with HBSS, pH7, was added, and the amount of $ONOO^-$ was measured by a chemiluminometer for 20 min. (B) Measurement of O_2^- by luminol-dependent chemiluminescence. Samples in 100 μl of Tris-HCl buffer, pH 7.4, containing 10 U/ml xanthine oxidase were incubated in a 96-well plate, and 1 μM of MCLA (50 μl) were added. Xanthine (30 μM, 50 μl) was added to generate O_2^-, and the amount of $ONOO^-$ was measured by a chemiluminometer for 20 min.



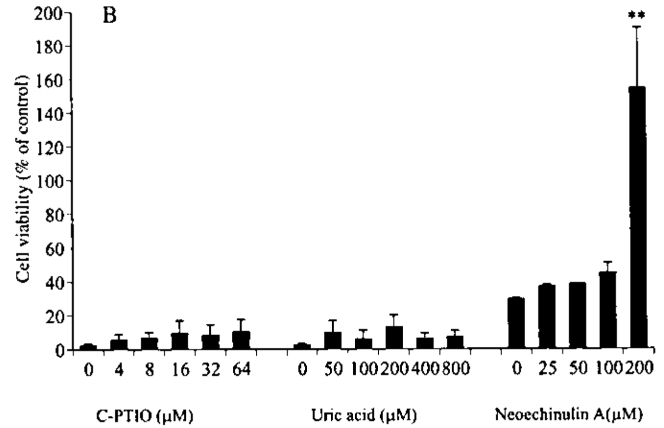


Fig. 6. **Neuroprotective effect of neoechinulin A and scavengers.** PC12 cells were seeded at a density of 3.0×10^3 cells/cm^2 and treated with 100 ng/ml NGF for 5 d. Cell viability was measured using a Cell Counting Kit-8. (A) Cells were incubated with scavengers for 24 h, and treated with 1 mM SIN-1 for 24 h. (B) Cells were incubated with neoechinulin A for 24 h. The neoechinulin A was removed and the cells were treated with 1 mM SIN-1 for 24 h. (means ± SD, $n = 4$) $^*p < 0.05$, $^{**}p < 0.01$.

from SIN-1. In addition, neoechinulin A specifically protects neuronal cells against SIN-1–induced cell death (Fig. 2). Thus, neoechinulin A is very useful for investigations of ONOO$^-$-induced neuronal cell death.

ONOO$^-$ is a powerful oxidant and cytotoxin whose production has been associated with conditions that result in damage to neurons. The appearance of nitrotyrosine immunoreactivity in postmortem brain from individuals with Parkinson's disease and other neurodegenerative conditions (*40–42*) also serves as indirect evidence of ONOO$^-$ production. It is well known that extracts of *Gingko biloba* leaves (EGb 761) rescue neuronal cells against ROS-induced cell death (*43*, 44). EGb 761 includes two major groups of constituents, flavonoids and terpenoids, that are involved in scavenging and anti-apototic activities. It has been reported that EGb 761 increases the level of mRNA for the mtDNA-encoded subunit 1 of NADH-dehydrogenase (*45*) and suppresses the activation of caspase-3 caused by various apoptosis inducers (*46*). In the case of neoechinulin A, one compound has scavenging, neurotrophic factor-like and anti-apoptotic activities. It should be noted that neoechinulin A scavenges ONOO$^-$, but not NO, because NO has various physiological functions (*4–7*). Therefore, neoechinulin A may be useful for protection against ONOO$^-$-induced neuronal cell death in neurodegenerative diseases.

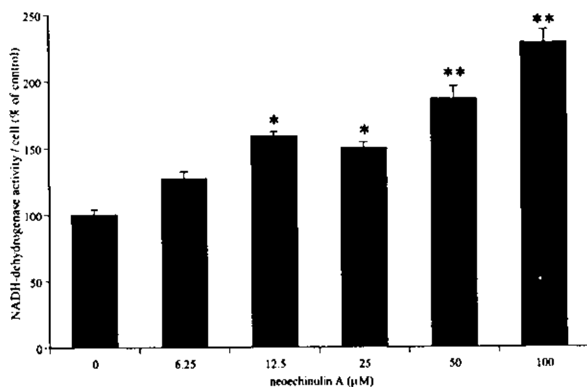
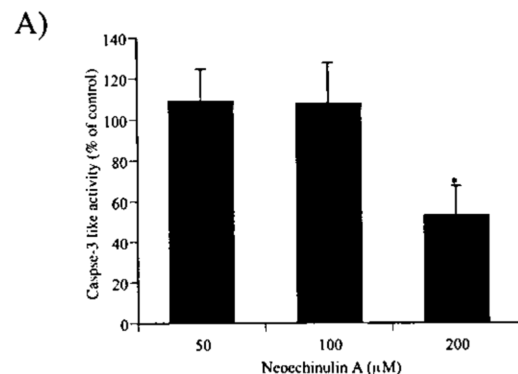


Fig. 7. **Effect of neoechinulin A on the NADH-dehydrogenase activity of PC12 cells.** PC12 cells were seeded at a density of 3.0×10^3 cells/cm^2 and treated with 100 ng/ml NGF for 5 d. The indicated concentration of neoechinulin A was added to each well and the relative NADH-dehydrogenase activity per cell was determined as described in "MATERIALS AND METHODS" (means ± SD, $n = 4$). $^*p < 0.05$, $^{**}p < 0.01$.



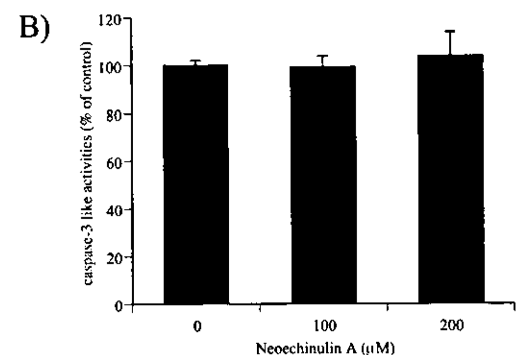


Fig. 8. **Effect of neoechinulin A on caspase-3 like protease activity.** PC12 cells (A) and 3Y1 cells (B) were seeded at a density of 3.0×10^3 cells/cm^2. PC12 cells were treated with 100 ng/ml NGF. Five days later, the cells were incubated with neoechinulin A for 24 h. After removal of the neoechinulin A, 0.5 mM SIN-1 was added. After 3 h, caspase activity was measured as described in "MATERIALS AND METHODS" (means ± SD, $n = 4$). $^*p < 0.01$.

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第15回 モノクローナル抗体を用いたポリグルタミン酸化チューブリンの神経細胞内局在

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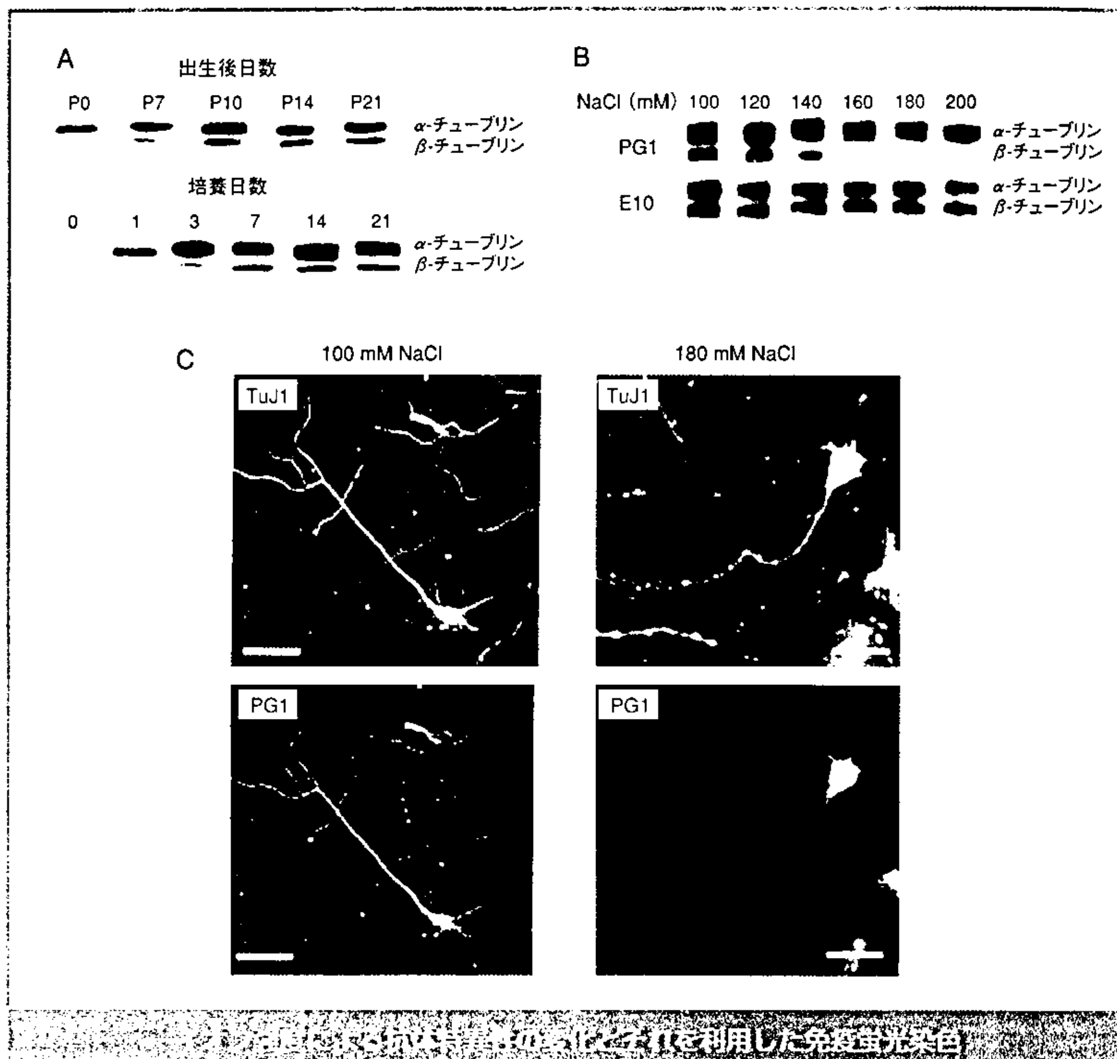

…それを利用した免疫蛍光染色

A）発達過程を追ったラット脳およびレチノイン酸存在下培養のラット胎仔脳神経幹細胞におけるチューブリンのポリグルタミン酸化を，抗ポリグルタミン酸化チューブリン抗体PG1により解析した．培養１日後には胎仔脳と同様にα-チューブリンが，３日目以降は成熟脳と同様にα-，β-両チューブリンがこの翻訳後修飾を受けた．B）PG1の反応特異性はNaCl濃度に依存し，高塩濃度ではα-チューブリン特異的であった．C）７日培養の神経幹細胞におけるこの修飾チューブリンの局在を，PG1と抗βIII-チューブリン抗体TuJ1を用いた二重免疫蛍光抗体法で調べた．100 mMと180 mM NaCl条件下における染色性の相違は，神経突起ではβ-チューブリンのみがポリグルタミン酸化されることを示している．スケールバー：50 μm

背　景

α-，β-チューブリンのC末端はグルタミン酸残基の豊富な領域であるが，この領域内のグルタミン酸残基のγ位カルボキシル基に，さらに数個のグルタミン酸残基が付加するポリグルタミン酸化という翻訳後修飾を受ける．モノクローナル抗ポリグルタミン酸化チューブリン抗体の多くは，α-とβ-チューブリンの両者を認識する[1]．多くの臓器や胎仔脳ではα-チューブリンのみがこの修飾を受けているのに対し，成熟脳では両チューブリンが修飾される[2]．現在のところ，この翻訳後修飾を受けたチューブリンの神経細胞内局在の研究は進んでおらず，この翻訳後修飾の果たす役割についての有力な考えも確立されていない．

イメージングが明らかにしたポリグルタミン酸化チューブリンの神経細胞内局在

モノクローナル抗ポリグルタミン酸化チューブリン抗体PG1は，図Aの上に示したように，ラット脳の発達の過程におけるα-，β-チューブリンのポリグルタミン酸化の制御を調べるうえで有用である．また，胎仔脳あるいは成熟脳から神経幹細胞を調製する技術や神経細胞に分化させる培養系は確立されている[3]．そこで，ラット胎仔脳神経幹細胞をレチノイン酸存在下において8ウェルカルチャースライド中で培養し，PG1を用いたウエスタンブロットによりチューブリンのポリグルタミン酸化を調べた．図Aの下に示したように，1日後にはα-チューブリンのみがポリグルタミン酸化され，3日目にはβ-チューブリンの修飾も認められた．β-チューブリンの修飾は，7日目でほぼ一定となった．このことは，この培養系がチューブリンポリグルタミン酸化の研究に有用であることを示している．一方，PG1のα-，β-チューブリンに対する特異性がリン酸緩衝液中のNaCl濃度により変化することを見出した（図B）．すなわち，100 mM NaCl中では両チューブリンを認識したが，180 mM NaCl濃度ではα-チューブリン特異的な反応性を示した．これに対し，同じ抗ポリグルタミン酸化チューブリン抗体E10の場合は，NaCl濃度による反応特異性の変化は起こらなかった．

レチノイン酸存在下で7日培養した神経幹細胞におけるポリグルタミン酸化チューブリンの局在を，この塩濃度変化に依存した抗体特異性の変化を利用して，PG1と神経細胞特異的な抗βⅢ-チューブリン抗体TuJ1を用いた二重免疫蛍光抗体法により観察したのが図Cである．100 mM NaClの染色条件下では細胞体と神経突起の両者が染色されたのに対し，180 mMにおいては細胞体しか染色されなかった．このことは，神経突起においてはβ-チューブリンのみがポリグルタミン酸化されることを示唆している．

翻訳後修飾タンパク質を認識するモノクローナル抗体には，タンパク質特異性の低いものが多い．塩濃度変化によりタンパク質特異性のある反応条件を見出したという本研究結果は，モノクローナル抗体が翻訳後修飾の研究にとって有力な武器となることを改めて示したものと意義づけることができる．

今後の展望と可能性

脳の発達に伴うチューブリンのポリグルタミン酸化の研究から，β-チューブリンのポリグルタミン酸化は神経細胞の成熟と関連すると考えられている．β-チューブリンのポリグルタミン酸化が神経突起において重要な役割を果たしていることを示唆するこの結果は，今後の研究の発展の契機となりうるものである．カチオン性リポソームを用いてモノクローナル抗体を細胞内に導入する技術が開発され，抗チューブリンモノクローナル抗体の導入が細胞増殖を阻害することが示されている[4]．イメージングとこれらの技術を併用することにより，機能解明が大きく進展することが期待される．

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● 使用機器 ●

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Inflammation Research

Histopathological features of murine systemic vasculitis caused by *Candida albicans* extract – an animal model of Kawasaki Disease

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Abstract. *Objective and Design*: We examined the histopathological features of systemic vasculitis caused in mice by injection of a *Candida albicans* (*C. albicans*) extract and investigated the principal genetic roles in the development of vasculitis.
Materials and Methods: C. albicans extract was injected intraperitoneally for five consecutive days in the 1st and 5th weeks to CD-1, C57BL/6N, C3H/HeN, BALB/cAnN, DBA/2N and CBA/JN mice. At week 8, mice were killed, and histological examination was performed by light microscopy.
Results: Arteritis had developed in 66% of CD-1 mice. The extramural coronary arteries and aortic root close to the orifice of coronary arteries were most frequently involved. Histologically, the characteristic feature of the arteritis was proliferative and granulomatous inflammation accompanied by numerous macrophages, lymphocytes, plasma cells and neutrophils. Fibrocellular intimal thickening with destruction of the internal elastic lamina and media was also observed. Five mouse strains after injection of *C. albicans* extract were clearly classified into a resistant group (CBA/JN, DBA/2N and BALB/cAnN mice) and a sensitive group (C3H/HeN and C57BL/6N mice). The inbred mouse strains which showed the same histocompatibility-2 (H-2) haplotype exhibited a different susceptibility to development of vasculitis.
Conclusion: This arteritis murine model shows unique histological features that have not been observed in other animal vasculitis models and it most closely resembles Kawasaki disease in humans. The genetic control of susceptibility to induction of vasculitis by the *C. albicans* extract is dependent to the mouse strains, but is not linked to the H-2 loci.

Key words: Systemic vasculitis – coronary arteritis – *Candida albicans* – Kawasaki disease

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Introduction

Kawasaki disease (KD) is an acute febrile illness that affects mainly infancy and early childhood. The most important complication is coronary arteritis leading to the formation of aneurysm. This disease has attracted special interest because it is most frequently attributed to ischemic heart disease in children caused by thrombosed coronary aneurysms [1, 2]. Although many studies have been performed, the etiology of this disease is not well understood. In addition to our ongoing histopathological study of Kawasaki disease autopsy patients, we established an interesting systemic vasculitis mouse model in which coronary arteritis was induced by intraperitoneal injection of *Candida albicans (C. albicans)* extract [3, 4]. This is a useful animal model of Kawasaki disease, since it exhibits many histological similarities to Kawasaki disease [3]. However, the histopathological changes seen in this vasculitis model have not been described in detail. Accordingly, we tried to clarify the histopathological features of the arterial lesions in this mouse model and to compare them with those in Kawasaki disease. In addition, we investigated the role of genetic factors in the development of vasculitis. Five inbred mouse strains were injected intraperitoneally with *C. albicans* extract, and the incidence of vasculitis and the histopathological features were compared.

Materials and methods

Preparation of C. albicans extract

C. albicans extract was prepared by the following technique. Yeast cells of a *C. albicans* strain (MCLS-6) that had been isolated from the feces of a child with Kawasaki disease were incubated without agitation at 37°C for 72 h in Sabouraud dextrose medium containing 2% glucose (MERCK, Germany). The yeast cells were harvested by centrifugation. An extract was obtained from the yeast cells using boiling water and 0.5 M KOH. After neutralization and dialysis against water for 3 days, the extracted material was precipitated with ethanol. The precipitate was suspended in phosphate-buffered saline and adjusted to a final concentration of 20 mg/ml.