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Korean mistletoe lectin (KML-IIU) and its subchains induce nitric oxide (NO) production in murine macrophage cells

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Abstract

Synthesis of nitric oxide (NO) is one of the important effector functions of innate immune cells. Although several reports have indicated mistletoe lectins induce immune cells to produce cytokines, studies regarding the activities of the lectins in the production of NO have been very limited. Here, we report on the induction of NO synthesis in a murine macrophage cell line, RAW264.7, by Korean mistletoe lectin (KML-IIU). When the macrophage cells were treated with KML-IIU in the presence of a suboptimal concentration of IFN-γ, NO production was induced in a concentration-dependent manner. Significantly higher levels of NO were induced by subchains of the KML-IIU (A and B), which have lower toxicities, as compared to the hololectin. Furthermore, expression of the inducible nitric oxide synthase (iNOS) gene was elevated in accordance with the level of NO production. When the synthase was inhibited by iNOS inhibitors (L-NIL and L-NAME), NO production was specifically reduced in a concentration-dependent manner. Our studies demonstrate that the KML-IIU and its subchains induce NO production in murine macrophage cells via activation of the iNOS gene expression, suggesting that the KML-IIU subchains may be used as an immunomodulator to enhance the effector functions of innate immune cells.

Introduction

Mistletoe lectin has become a subject of interest due to its marked biological activities including its cytotoxicity and immunomodulatory effects [1–9]. Mistletoe lectin consists of two subunits, an A chain and a B chain, linked by a disulfide bond. The A chain is cytotoxic to various cancer cells while the B chain helps the A chain bind and enter into the target cells [4, 10]. The disulfide bridge between the A chain and the B chain is necessary for maintaining the cytotoxicity of the lectin [11].

Numerous studies, both in vitro and in vivo, have shown that mistletoe lectin induces immune cells to produce inflammatory cytokines, such as TNF-α, IFN-γ, IL-1, IL-12 and IL-6 [7, 12, 13]. Not only does the mistletoe hololectin (a complex of A and B subchains linked by a disulfide bond) induce cytokine release, individual subchains of the mistletoe lectin also do the same [7]. As for murine macrophages, the A chain is better at inducting expression of TNF-α mRNA than the B chain [14]. These findings indicate that the mistletoe lectin can be a potent activator of immune

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cells. Nitric oxide (NO), a gaseous free radical, is known to be one of the most important effector molecules of innate immune cells. Its production is stimulated in macrophages when the cells are exposed to LPS, TNF-α, or IFN-γ [15, 16]. Nitric oxide is synthesized by three different kinds of NO synthases: iNOS, eNOS, and nNOS [17–19]. It mediates vasodilatation, inflammation, immune responses, and even platelet aggregation. These biological activities actually play significant roles in antibacterial, antiparasital, and tumoricidal activities in mice [20–22]. In addition, the NO in innate immune cells is essential to the improvement of tuberculosis in mice [23, 24] and in human [23, 25].

Although Korean mistletoe lectin has demonstrated its immunomodulatory effects via in vitro as well as in vivo studies as explained earlier, the possibility of NO induction by the lectin has not yet been explored. Therefore, we investigated activities of the Korean mistletoe lectin (KML-IIU) and its subchains in NO production using a murine macrophage cell line, RAW264.7. The results indicate that mistletoe lectin and both of it's A and B subchains increase NO synthesis from macrophage cells by activating an expression of the iNOS gene.

Materials and methods

Separation and isolation of subchains from KML-IIU

Korean mistletoe lectin (KML-IIU) was purified as previously described elsewhere [26, 27]. The purification process was performed under acidic conditions (3% acetic acid) in order to eliminate or significantly reduce any possible endotoxin effect. To separate subchains A and B, 5 mg of KML-IIU hololectin was dissolved in 15 ml of reducing buffer [10 mM phosphate buffered saline (PBS; pH 7.4) containing 5% of 2-mercaptoethanol] and was incubated at 4°C for 12 h. The sample was then applied to a HCl-hydrolyzed Sepharose 4B column (3×9 cm, 50 ml), and the column was then washed with a 5-fold column volume of the reducing buffer. Subchain A was eluted while subchain B was attached to the column resin. The B chain was later eluted with 0.1 M lactose solution. The protein fractions were pooled and were dialyzed extensively against PBS, and their concentrations were determined by BCA assay [28].

SDS-polyacrylamid gel electrophoresis (PAGE)

SDS-PAGE was carried out in a 12.5% separation gel under a reducing condition (2.5% mercaptoethanol, 2-ME) as described elsewhere [29].

Hemagglutination assay

Serially diluted lectin samples (two-fold dilution) were applied into a U-type microtiter plate containing 2% human erythrocytes (blood group B) in a 10 mM sodium-phosphate buffer (pH 7.4). Following a brief mixing, the erythrocytes were allowed to settle for 1 h at room temperature in order to determine their agglutination. Agglutinating red blood cells settled as a film at the bottom of the well whereas unagglutinating cells formed a button shape at the bottom.

Cell line and reagent

RAW264.7 cells, a murine macrophage cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco BRL) and 100 U/ml of penicillin-streptomycin (Gibco BRL) in a humidified atmosphere with 5% CO_2 at 37°C.

Murine recombinant interferon-γ (IFN-γ) and LPS were purchased from Roche Diagnostics (Mannheim, Germany) and Sigma (Missouri, USA), respectively. Inhibitors of NO synthase, L-N6-(1-Iminoethyl) Lysine (L-NIL) and NG-Nitro-L-Arginine-Methyl Ester Hydrochloride (L-NAME) were purchased from Sigma.

Cytotoxicity test

RAW264.7 cells (1×10^5 cells/well) in 96-well plates were incubated with the indicated concentrations of the lectin samples for 24 h. The cell viability was measured by XTT method as described elsewhere [30].

Cell stimulation

Cells were seeded in 24-well plates (Nunc) at a density of 5×10^5 cells/well in DMEM supplemented with 10% FBS. Following incubation for 3 h at 37°C under 5% CO_2, the cells were added with various concentrations of the lectins plus 50 U/ml of IFN-γ and further cultivated. After

24 h incubation, the supernatants were collected and stored at −20°C until assayed for NO production.

Measurement of NO concentration

Nitric oxide production in cell cultures was measured as previously described [31]. In short, a small portion (100 μl) of the cell culture supernatant was incubated at room temperature for 10 min with an equal volume of the Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrchloride, and 2.5% H3PO4). Nitric oxide concentration was determined by measuring nitrites in the mixture through an ELISA reader (Bio-Tek, Sweden) at 540 nm wave length.

RNA extraction and Northern blot analysis

After the RAW264.7 cells stimulated by the mistletoe lectin for 12 h, the total cellular RNA was extracted using Trizol (Gibco BRL) according to the provider's instructions. The RNA samples, transferred to a nylon membrane (Roche, Mannheim, Germany), were analyzed by hybridization with the mouse iNOS-specific oligoprobe labeled with digoxigenin (Sigma-Genosys Ltd., Cambridge, UK). The hybridized oligoprobe was then detected by the anti-digoxigenin antibodies conjugated with alkaline phosphatase (Roche, Mannheim, Germany). The amount of RNA loaded on the gel for the Northern blot analysis was assessed by hybridization with a 18S ribosomal RNA-specific probe [32].

Results

Isolation of a lectin (KML-IIU) from the Korean mistletoe and obtaining its subchains

The lectin KML-IIU was purified from the Korean mistletoe and the hololectin was processed to obtain its A and B subchains as described above [26, 27]. Following treatment with 2% β-mercaptoethanol overnight at room temperature, the KML-IIU mixture was applied to a hydrolyzed Sepharose 4B column. While the A chain passed through the column, the B chain was bound to the column. The B chain was then eluted with 0.1 M lactose. Figure 1 shows the KML-IIU hololectin isolated along with its subchains as assessed by 12.5% SDS-PAGE. Molecular weights of the A and the B subchains were estimated to be 30 Kd and 34 Kd, respectively. Only the B chain, but not the A chain, agglutinated erythrocytes as expected (Figure 2). These results suggest that both the A and the B subchains were obtained in an active form.

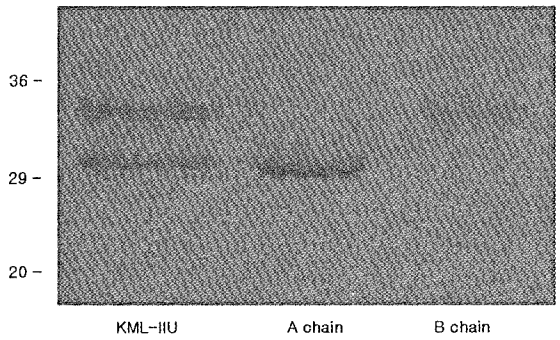

Figure 1. SDS-PAGE analysis of KML-IIU and its subchains under reducing condition. The proteins were separated by 12.5% SDS-PAGE and stained with Coomassie blue.

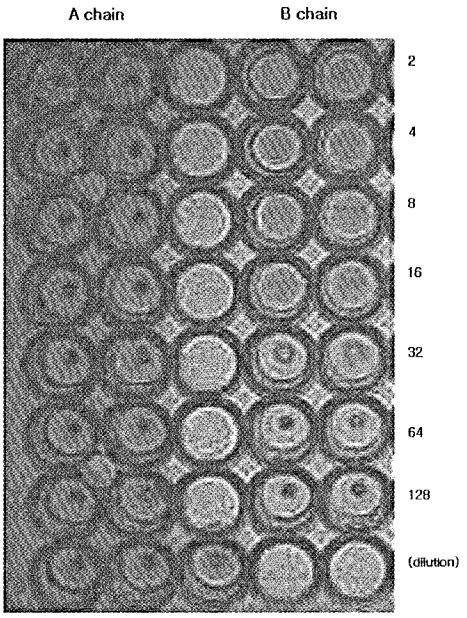

Figure 2. Hemagglutination of human erythrocytes (type B) treated with the subchain A or the B as derived from the KML-IIU hololectin. Fifty μl of samples in two-fold serial dilution were mixed with an equal volume of erythrocyte suspension (2% in PBS) and incubated for 1 h at room temperature. Untreated erythrocytes were used as a control.

Cytotoxicity of KML-IIU and its subchains

In order to investigate the cytotoxicity of KML-IIU and its subchains, RAW264.7 murine macrophage cells were incubated for 24 h in the presence of different concentrations of the lectins. As indicated in Figure 3, the hololectin showed much stronger cytotoxic activity than the subchains even at a 40 ng/ml level, which is consistent with the previous studies [26]. However, subchain B showed a moderate cytotoxicity at 1000 ng/ml while subchain A was nontoxic to the cells at the same concentration.

Induction of NO production by KML-IIU and its subchains

As shown in Figure 4, NO production was markedly increased in a concentration-dependent manner when macrophage cells, RAW264.7, were treated either with KML-IIU, with subchain A, or with subchain B in combination with IFN-γ (50 U/ml). The KML-IIU holoenzyme showed a decreased NO production at concentrations higher than 40 U/ml. When the cells were treated with less than 40 ng/ml of the lectin, the level of NO induced was inconsistent (data not shown).

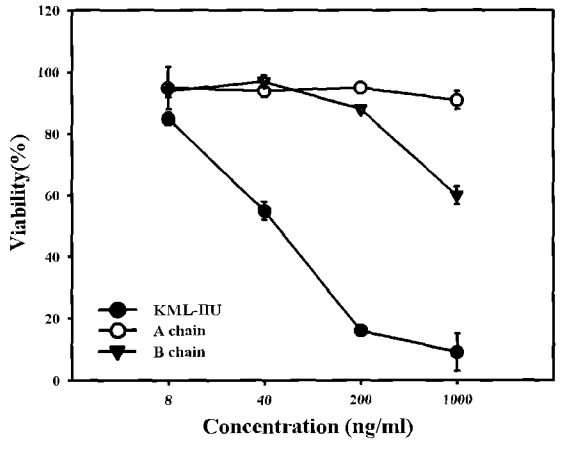

Figure 3. Cytotoxic effect of KML-IIU and its subchains. Cells (1×10^5 cells/well) were incubated with the indicated concentrations of samples for 24 h in 96-well plates. The cell viability was measured via the XTT method. The data were represented as the mean + SD in triplicate.

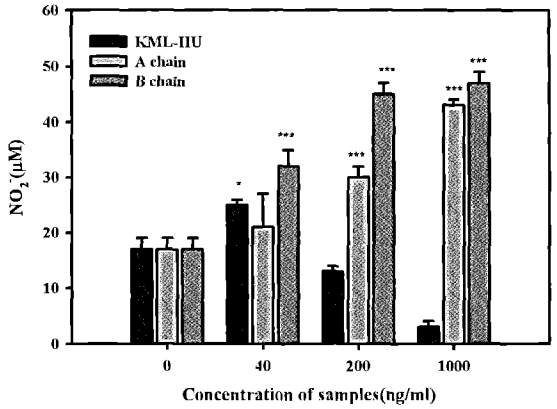

Figure 4. Concentration-response effects of KML-IIU and its subchains on NO production in RAW264.7 cells. Cells (5×10^5 cells) were plated in 24-well plates and stimulated with the indicated concentrations of lectin samples plus IFN-γ (50 U/ml) for 24 h. Nitric oxide release was measured by the Griess method and represented as the mean + SD in triplicate. The symbols * and *** indicate the statistical P values <0.01 and <0.001, respectively, as compared to the group treated with IFN-γ alone.

Inhibition of lectin-induced NO production by iNOS-specific inhibitors

To investigate whether the NO production induced by the KML-IIU or its subchains is involved in the L-Arginine-dependent pathway, the RAW264.7 macrophage cells were incubated for 1 h in the presence of NO synthase inhibitors: L-N6-(1-Iminoethyl)Lysine (L-NIL), or NG-Nitro-L-Arginine-Methyl Ester Hydrochloride (L-NAME) [33–35]. The NO production was progressively inhibited by L-NIL and L-NAME in a concentration-dependent manner (Table 1). However, inhibition of NO production by L-NIL was more efficient compared to L-NAME.

Activation of the iNOS gene expression by the mistletoe lectin, KML-IIU

To further confirm if the increased NO production is correlated with the level of iNOS gene expression, the level of iNOS mRNA in the lectin-treated macrophage cells was determined by Northern blot analysis. As shown in Figure 5, iNOS mRNA was not expressed when the RAW264.7 cells were treated with IFN-γ alone. Even when the cells were treated with the KML-IIU hololectin in combination with IFN-γ, the iNOS gene was induced

Table 1. Inhibition of NO production induced by KML-IIU or its subchains by iNOS-specific inhibitors.

Inhibitors	Media alone	LPS	KML-IIU	A chain	B chain
–	19 ± 1	68 ± 6	23 ± 2	42 ± 1	45 ± 2
NIL(mM)					
1	5 ± 1	18 ± 2	6 ± 1	11 ± 1	10 ± 1
0.1	14 ± 4	42 ± 1	16 ± 2	26 ± 1	23 ± 4
0.01	16 ± 1	64 ± 2	23 ± 3	41 ± 1	31 ± 2
NAME					
1 mM	5 ± 3	42 ± 1	9 ± 1	18 ± 1	22 ± 1

RAW264.7 cells (5×10^5 cells) were pre-incubated for 2 h with NIL or NAME. The cells were then treated with the mistletoe lectin or its subchains plus IFN-γ and cultured further for 24 h. Nitric oxide release was measured by the Griess method and represented as the mean + SD in triplicate.

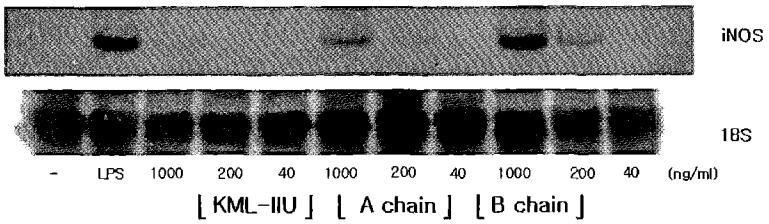

Figure 5. The levels of iNOS mRNA in RAW264.7 cells treated KML-IIU and its subchains. The cells (2.5×10^6 cells) were treated with KML-IIU or its subchains in the presence of 100 U/ml IFN-γ. After 12 h incubation, the cells were lysed and the total cellular RNA isolated. The presence of iNOS mRNA was detected by Northern blot analysis with an iNOS-specific probe. RNA loading of the gel for Northern blot analysis was assessed by hybridization with an 18S ribosomal RNA-specific probe.

slightly. However, expression of iNOS mRNA was apparently induced, in a concentration-dependent manner, in the cells treated with either the subchain A or the subchain B plus IFN-γ. The B chain induced better expression of the iNOS mRNA as compared to the A chain.

Discussion

Many studies have suggested that mistletoe lectin activates monocytes and macrophages for secretion of cytokines, such as TNF-α, IL-1, and IL-6 [36, 37]. The two subchains of the lectin were shown to have similar functions as well [7, 14, 38]. However, studies concerning activities of the mistletoe lectin on production of NO, a major effector molecule of activated innate immune cells, are very limited. In particular, an effect of the subchains of mistletoe lectin on NO production has not yet been reported.

In this paper, we report that Korean mistletoe lectin, KML-IIU, as well as its subchains A and B, induced NO production from RAW264.7 macrophage cells via activation of iNOS gene expression. When the RAW264.7 macrophage cells were treated with the hololectin KML-IIU, NO production was slightly induced at 40 ng/ml. Both A and B subchains of the lectin, however, significantly induced NO production in a concentration-dependence manner. It has been well documented that NO produced from macrophages contributes to the control of tuberculosis or to its associated pathology in humans [39]. The macrophage NO is also required to control the same disease [40] as well as malaria [41] in mice. S-nitrothiols are the most important active forms of NO as an antimicrobial agent [42]. NO could act in synergy by reacting with O_2 H_2O_2, · OH, and sometimes HOCl produced by monocytes and activated macrophages [43]. In addition, macrophage NO is important for antitumor activities. Inhibition of NO production by administration of NOS inhibitors to mice has promoted growth of UV light-induced skin cancers [44] and increased NO production by transfecting NOS cDNA significantly reduced melanoma cell proliferation and metastasis [45].

Among three genes encoding NO synthases (nNOS, eNOS, and iNOS), iNOS only appears to express in primary macrophages [46]. The iNOS is, therefore, a major resource for production of macrophage NO, which is responsible for antitumor and antibacterial functions. The level of iNOS gene expression was directly correlated with the concentration of NO released in the media from the cells treated with the lectin. When competitive inhibitors of iNOS (L-NIL and L-NAME) were added to the lectin-induced cells, the NO production was specifically inhibited in a concentration-dependent manner. These results clearly indicate KML-IIU and its subchains induce NO production from the macrophages through augmented expression of iNOS gene. Our finding supports a previous report that mice with mammary adenocarcinoma were shown an increased NO production in the blood when treated with IL-2 and the European mistletoe lectin, i.p, for immunotherapy [47].

The subchains of KML-IIU were less or non-toxic to the cells as compared to the hololectin. It has been known that the A chain of mistletoe lectin enters into the target cell with the help of the B chain and inhibits protein synthesis of the cell by directly binding to a translation factor [48, 49]. The A chain alone cannot enters into the target cells without the B chain's help [11]. The lower toxicity of the subchains may be due to the inability of those subchains to enter target cells alone. Both the A and the B subchains induced NO production much better than the hololectin. This result may be due to the higher toxicity of the hololectin, which results in a reduction in cell numbers and a consequent lowering of NO production. However, this explanation may not apply to cases where the lectin concentration is 40 ng/ml or less, which showed only minor cytotoxicity. Although the KML-IIU hololectin and subchain A were comparable in NO production, subchain B showed higher NO inducibility (approximately 40% higher) than the others at the same concentration. If the lectin concentration was reduced less than 40 ng/ml, induced NO production was inconsistent. The underlying mechanism whereby the subchains induce NO production better than the hololectin is not clear at present. It is hypothesized that the subchains in a free form may have easier access to a receptor(s) on the cell surface related to the activation of the iNOS gene. Further studies are required to confirm the possibility.

In conclusion, we demonstrate that Korean mistletoe lectin, KML-IIU, and it subchains induce NO production via activation of the iNOS gene expression. The subchains of the KML-IIU, which showed lower cytotoxicity and higher NO inducement, may be good candidates for a potent immunomodulator to enhance the effector functions of innate immune cells.

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