Induction of Nitric Oxide Synthase by *Oldenlandia diffusa* in Mouse Peritoneal Macrophages

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Oldenlandia diffusa **(OD) has been used to treat malignant tumors. In this study using mouse peritoneal macrophages, we have examined the mechanism by which OD regulates nitric oxide (NO) production. When OD (1 mg/ml) was used in combination with 10 U/ml of recombinant interferon-γ (rIFN-γ), there was a marked cooperative induction of NO production (36.13±7.12 μM) by the Griess method (nitrite). Treatment of macrophages with rIFN-γ plus OD (1 mg/ml) caused a significant increase in tumor necrosis factor-α (TNF-α) production (4.49±1.43 ng/ml) by enzyme-linked immunosorbent assay. The increased production of NO and TNF-α from rIFN-γ-plus OD-stimulated cells was almost completely inhibited by pretreatment with 100 μM of pyrrolidine dithiocarbamate (PDTC), an inhibitor of nuclear factor kappa B (NF-κB). PDTC also inhibited phosphorylation of IκB in rIFN-γ-plus OD-stimulated cells. These findings demonstrate that OD increases the production of NO and TNF-α by rIFN-γ-primed macrophages and suggest that NF-κB plays a critical role in mediating these effects of OD.**

Key words *Oldenlandia diffusa*; nitric oxide; peritoneal macrophages; tumor necrosis factor-α

Oldenlandia diffusa (OD, Rubiaceae), an herbal medicine, has been used in traditional Oriental medicine for treating liver, lung, and rectal tumors. Previous investigations showed that Oldenlandiae diffusae Herba and its major compound, ursolic acid, has anticancer effects[1] and that Oldenlandiae diffusae Herba inhibited mutagenesis, tumor growth *in vivo*,[2] DNA binding, and metabolism of aflatoxin B1,[3,4] and benzo(a)pyrene bioactivated by Aroclor 1254 (polychlorinated biphenyl)-induced rat liver 9000×g supernatant (S9).[5]

Nitric oxide (NO) is a highly reactive molecule produced from a guanidino nitrogen of arginine by NO synthase (NOS) enzymes.[6] Over the past decade, NO as a potent macrophage-derived effector molecule against a variety of bacteria, parasites, and tumors has received increasing attention.[7] Original evidence of tumor cell cytostasis and cytotoxity was found in macrophage-tumor cell cocultures in which cytokine- and/or lipopolysaccharide (LPS)-stimulated macrophages inhibited metabolic functioning of cocultured tumor cells.[8] The expression of NO has been linked with DNA damage, thus stimulating the expression of wild-type p53 and ultimately leading to apoptosis.[9] NO may also be induced in target cells themselves, resulting in apoptotic cell death induced by autoexpression of inducible NOS (iNOS).[10]

The proinflammatory cytokine tumor necrosis factor-α (TNF-α) regulates systemic responses to microbial infection or tissue injury.[11] These signals stimulate immune functions and induce expression of acute-phase reactants in the liver, among other effects. Production of TNF-α protein is enhanced by the presence of interferon-γ (IFN-γ). TNF-α then acts as an autocrine signal to amplify IFN-γ-induced production of NO in macrophages.[12]

Macrophages are a major source of cytokines such as TNF-α, and induction of cytokine gene expression by LPS occurs primarily at the level of transcription and involves the action of several transcription factors, including members of the nuclear factor-κB (NF-κB)/rel, C/EBP, Ets, and AP-1 protein families.[13] In particular, NF-κB bound to specific consensus DNA elements present on the promoters of target genes initiates the transcription of TNF-α, iNOS, cyclooxygenase-2, and interleukin-6 (IL-6).[14]

OD has been used to treat malignant tumors and it stimulates the reticuloendothelial system.[15] We hypothesized that the antiinfectious and antitumor effects of OD are due to its ability to enhance the nonspecific immunologic function of NO.

In the present study, we show that OD synergistically induces NO and TNF-α production by peritoneal macrophages when the cells are treated with recombinant IFN-γ (rIFN-γ). To investigate the mechanism of OD-induced NO and TNF-α production, we examined the ability of NF-κB inhibitors such as pyrrolidine dithiocarbamate (PDTC) to block the OD-induced effect. PDTC decreased NO and TNF-α production induced by rIFN-γ plus OD. These findings may explain how OD influences NO and TNF-α production *via* the NF-κB signaling pathway.

MATERIALS AND METHODS

Reagents Murine rIFN-γ (1×10^6 U/ml) was purchased from Genzyme (München, Germany). Dulbecco's Modified Eagle's Medium (DMEM), *N*-(1-naphtyl)-ethylenediamine dihydrochloride, LPS, sodium nitrite, PDTC, and 3-[4,5-dimetylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, U.S.A.). Rabbit polyclonal antisera to iNOS were obtained from Transduction Laboratories (Lexington, KY, U.S.A.). Phosphated-IκBα (p-IκBα) antibody was purchased from New England Bio-

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labs (Beverly, MA, U.S.A.). Recombinant TNF-α (rTNF-α), biotinylated TNF-α, and anti-murine TNF-α were purchased from R & D System Inc., U.S.A. N^G-monomethyl-L-arginine (*N*MMA) was purchased from Calbiochem (San Diego, CA, U.S.A.). Thioglycollate (TG) was purchased from Difco Laboratories (Detroit, MI, U.S.A.). Syringe filters (0.2 μm), 4-well and 96-well tissue culture plates, and 100-mm diameter dishes were purchased from Nunc (Naperville, IL, U.S.A.). DMEM containing L-arginine (84 mg/l), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Life Technologies (Grand Island, NY, U.S.A.). Male C57BL/6 mice were purchased from Dae Han Experimental Animal Center (Eumsung, Republic of Korea).

Peritoneal Macrophage Cultures TG-elicited macrophages were harvested 3—4 d after injection of TG 2.5 ml i.p. to the mice and isolated as reported previously.[16] Using 8 ml of HBSS containing 10 U/ml of heparin, peritoneal lavage was performed. Then the cells were distributed in DMEM, which was supplemented with 10% heat-inactivated FBS, in 4-well tissue culture plates (2.5×10^5 cells/well) incubated for 3 h at 37 °C in an atmosphere of 5% CO_2, washed three times with HBSS to remove nonadherent cells, and equilibrated with DMEM that contained 10% FBS before treatment.

Preparation of OD The plant sample was obtained from the Oriental Drug Store, Duckhyun Dang (Iksan, Republic of Korea). An extract of OD was prepared by decocting the dried prescription of herbs with boiling distilled water. The duration of decoction was about 3 h. The decoction was filtered, lyophilized, and stored at 4 °C. The yield of dried extract from starting materials was about 8%. Dilutions were made in saline and then filtered through a 0.2-μm syringe filter.

Measurement of Nitrite Concentration Peritoneal macrophages (2.5×10^5 cells/well) were cultured with rIFN-γ (10 U/ml) for 6 h. The cells were then stimulated with various concentrations of OD. NO synthesis in cell cultures was measured using a microplate assay method, as previously described.[17] To measure nitrite, 100-μl aliquots were removed from conditioned medium and incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphtyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined in a Titertek Multiskan (Flow Laboratories, North Ryde, Australia). NO_2^- was determined by using sodium nitrite as a standard. The cell-free medium alone contained 5 to 8 μM of NO_2^-. This value was determined in each experiment and subtracted from the value obtained from the medium with peritoneal macrophages.

Assay of TNF-α Release Peritoneal macrophages (2.5×10^5 cells/well) were incubated with rIFN-γ (10 U/ml), OD, rIFN-γ plus LPS (10 μg/ml), and rIFN-γ plus various concentrations of OD for 24 h. Then the amount of TNF-α secreted by the cells was measured using a modified enzyme-linked immunosorbent assay (ELISA), as described previously.[18] The ELISA was devised by coating 96-well plates containing murine monoclonal antibody with specificity for TNF-α. Before use and between subsequent steps in the assay, coated plates were washed with phosphate-buffered saline (PBS) containing 0.05% Tween-20. All reagents used in this assay were incubated for 2 h at 37 °C. The rTNF-α was diluted and used as a standard. Serial dilutions starting from 1 pg/ml were used to establish the standard curve. Assay plates were exposed sequentially to alkaline-phosphatase-conjugated goat anti-rabbit IgG. Optical density readings were made within 10 min of the substrate on a Titertek Multiskan with a 405-nm filter. Appropriate specificity controls were included.

Western Blot Analysis Peritoneal macrophages (5×10^6 cells/well) were incubated for 6 h with rIFN-γ (10 U/ml). The cells were then stimulated with OD (1 mg/ml) or LPS (10 μg/ml) for 12 h. Whole-cell lysates were made by boiling peritoneal macrophages in sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol, and 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated by 7% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The membrane was then blocked with 5% skim milk in PBS-Tween-20 for 1 h at room temperature and then incubated with anti-iNOS and p-IκBα antibodies. After washing in PBS-Tween-20 three times, the blot was incubated with secondary antibody for 30 min and the antibody-specific proteins were visualized using the enhanced chemiluminesence detection system according to the recommended procedure (Amersham Corp., Newark, NJ, U.S.A.).

Determination of Endotoxin OD extracts used in this experiment were found to be less than 10 pg/ml of endotoxin as determined within the limits of assay E-TOXATE kit (Sigma), performed according to manufacturer's protocol.

Assay of MTT To investigate the viability of cancer cells, MTT assay was performed. Briefly, 500 μl of HepG2 human hepatoma cells (ATCC-HB-8065, Rockville, MD, U.S.A.) or HT-29 cells human colon cancer cell, (ATCC-HTB-38, Rockville, MD, U.S.A.) in suspension (2.5×10^4 cells) were cultured in 4-well plates for 24 h after treatment with each concentration of OD. Twenty microliters of MTT solution (5 mg/ml) was added and the cells were incubated at 37 °C for an additional 4 h. The crystallized MTT was dissolved by the addition of dimethyl sulfoxide 500 μl to the well. The amount of dark blue formazan was determined by measuring the absorbance at 570 nm.

Statistical Analysis Results were expressed as the mean$\pm$S.E.M. of independent experiments, and statistical analysis was performed using the Student's *t*-test to express the difference between two groups.

RESULTS

Effects of OD on NO Production in Activated Peritoneal Macrophages To determine the effect of OD on the production of NO by mouse peritoneal macrophages, we treated nonprimed (resting) and rIFN-γ-primed cells with OD. The resultant NO production was determined by detecting nitrite concentrations in the cell supernatants after 48 h treatment. As shown in Fig. 1, only OD had little effect on NO production in resting mouse peritoneal macrophages. However, when mouse peritoneal macrophages were primed for 6 h with murine rIFN-γ and then treated with various concentrations of OD (0.01—1 mg/ml), NO production was increased compared to nonprimed conditions.

Effects of OD on rIFN-γ-Primed iNOS Expression Data in Fig. 2A show the effects of rIFN-γ plus OD treat-

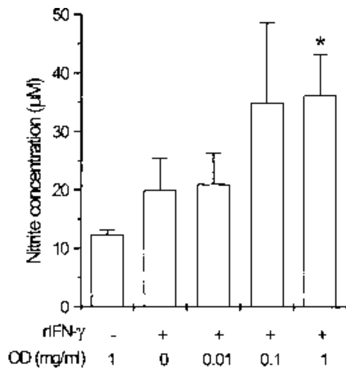

Fig. 1. Dose-Dependent Effects of OD on NO Synthesis in rIFN-γ Treated Peritoneal Macrophages

Peritoneal macrophages (2.5×10^5 cells/well) were cultured with rIFN-γ (10 U/ml). The peritoneal macrophages were then stimulated with various concentrations of OD for 6 h after incubation. After 48 h of culture, NO release was measured by the Griess method (nitrite). NO (nitrite) released into the medium is presented as the mean$\pm$S.E.M. of three independent experiments duplicated in each run. $*p<0.05$ compared with rIFN-γ alone.

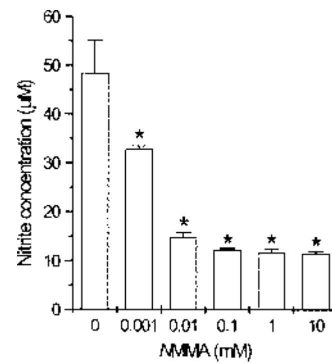

Fig. 3. Effects of *N*MMA on OD-Induced Nitrite Accumulation in the Cultured Medium of Peritoneal Macrophages

Peritoneal macrophages (2.5×10^5 cells/well) were incubated for 6 h with rIFN-γ (10 U/ml) plus various concentrations of *N*MMA. The peritoneal macrophages were then treated with OD (1 mg/ml) and cultured for 48 h. NO release was measured by the Griess method (nitirite). NO (nitrite) released into the medium is presented as the mean$\pm$S.E.M. of three independent experiments duplicated in each run. $*p<0.05$ compared with control (absence of *N*MMA).

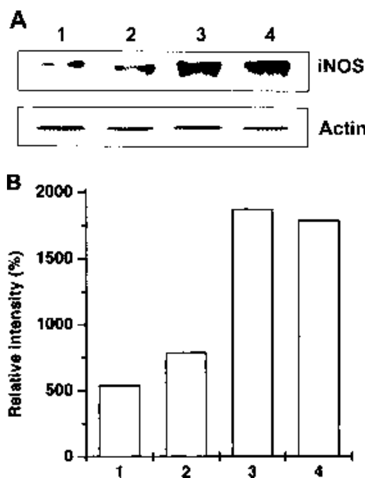

Fig. 2. Effects of OD on the Expression of iNOS by rIFN-γ Plus OD-Induced Peritoneal Macrophages

Peritoneal macrophages (5×10^6 cells/well) were incubated for 6 h with rIFN-γ (10 U/ml). The peritoneal macrophages were then stimulated with OD (1 mg/ml) or LPS (10 μg/ml) for 12 h. The protein extracts were prepared, and then samples were analyzed for iNOS expression by Western blotting as described in the Methods (A). The iNOS levels were quantitated by densitometry (B). 1, control; 2, rIFN-γ; 3, rIFN-γ+LPS; 4, rIFN-γ+OD.

Table 1. Effects of PDTC on rIFN-γ Plus OD-Induced NO Production in Peritoneal Macrophages

Addition		PDTC (μM)	Nitrite concentration (μM)[b]
rIFN-γ (10 U/ml)	OD (1 mg/ml)[a]		
−	−	−	10.1±0.6
+	−	−	19.8±5.5
+	+	−	36.1±7.1
+	+	1	33.22±2.13
+	+	10	13.73±0.58[c]
+	+	50	10.28±0.09[c]
+	+	100	9.58±0.18[c]

a) Peritoneal macrophages (2.5×10^5 cells/well) were stimulated with rIFN-γ plus OD or rIFN-γ plus OD plus various concentrations of PDTC. *b*) The amount of nitrite released by peritoneal macrophages was measured after 48 h using the Griess method. Values are the mean$\pm$S.E.M. of three independent experiments duplicated in each run. *c*) $p<0.01$ compared with rIFN-γ plus OD.

ment on the expression of iNOS protein in mouse peritoneal macrophages. rIFN-γ plus OD synergistically increased the expression of iNOS protein in mouse peritoneal macrophages. In Fig. 2B, the iNOS synthesis shown in Fig. 2A is normalized to the control value using an Image Master program (Pharmacia Biotech).

Inhibition of OD-Induced NO Production by *N*MMA *N*MMA is a specific inhibitor of NO production in the L-arginine-dependent pathway.[19] To determine whether the signaling mechanism in OD-induced NO production participates in the L-arginine-dependent pathway in mouse peritoneal macrophages, the cells were incubated for 6 h in the presence of rIFN-γ plus *N*MMA. The production of nitrite by rIFN-γ plus OD in mouse peritoneal macrophages was progressively inhibited with increasing amounts of *N*MMA. The OD-induced accumulation of nitrite was significantly blocked by *N*MMA (0.001—10 mM) (Fig. 3). *N*MMA did not inhibit iNOS expression (data not shown) as previously reported.[20]

Inhibition of OD-Induced NO Production by PDTC It is known that PDTC, an antioxidant compound, inhibits NF-κB activation.[21] As an approach to define the signaling mechanism of OD in NO production, we examined the influence of PDTC in rIFN-γ- plus OD-treated mouse peritoneal macrophages. Adding PDTC (1—100 μM) to the rIFN-γ- plus OD-treated mouse peritoneal macrophages decreased NO production in a dose-dependent manner (Table 1).

Effects of OD on rIFN-γ-Induced TNF-α Production The synergistic cooperative effect of OD on rIFN-γ-induced TNF-α production was examined. Mouse peritoneal macrophages secreted very low levels of TNF-α after 24-h incubation with medium alone or rIFN-γ alone. However, OD in combination with rIFN-γ markedly increased TNF-α production in a dose-dependent manner (Fig. 4). We also investigated the effect of OD on the signal transduction pathway of TNF-α production. Many actions of TNF-α can be ascribed to its ability to activate the transcription factor NF-κB.[22] Disruption of NF-κB-activating pathways can be envisioned as a means to influence various immune response conditions. As shown in Fig. 4, adding the NF-κB inhibitor

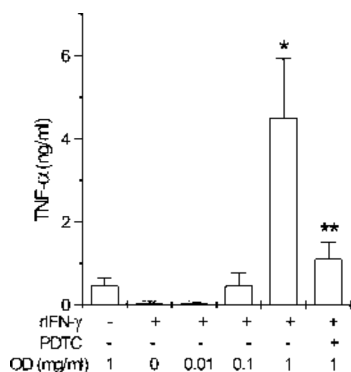

Fig. 4. Effects of OD on the Production of TNF-α by rIFN-γ Plus OD or rIFN-γ Plus OD Plus PDTC in Peritoneal Macrophages

Peritoneal macrophages (2.5×10^5 cells/well) were stimulated with rIFN-γ (10 U/ml) plus various concentrations of OD or rIFN-γ plus OD (1 mg/ml) plus PDTC (100 μM). The amount of TNF-α secreted by peritoneal macrophages was measured using the ELISA method after 24-h incubation. Values are the mean$\pm$S.E.M. of five independent experiments duplicated in each run. $*p<0.05$ compared with rIFN-γ alone; $**p<0.05$ compared to rIFN-γ+OD (1 mg/ml).

Fig. 5. Inhibition of OD-Induced Phosphorylation of IκBα by PDTC

Peritoneal macrophages (3×10^6 cells/dish) were stimulated with rIFN-γ (10 U/ml) plus OD (1 mg/ml) or rIFN-γ plus OD plus PDTC (100 μM) for 60 min. Cytosol protein was prepared and analyzed for phosphorylation of IκBα by Western blotting as described in the Methods. 1, control; 2, rIFN-γ+OD; 3, rIFN-γ+OD+PDTC.

PDTC to the rIFN-γ- plus OD-treated mouse peritoneal macrophages decreased the synergistic effects of OD on TNF-α production significantly.

Inhibition of OD-Induced Phosphorylation of IκBα by PDTC The translocation of NF-κB to the nucleus is preceded by the proteolytic degradation of IκBα.[23] Degradation of IκBα, the inhibitory protein bound to NF-κB, is a key step required for the activation of NF-κB. This process is initiated through signal-induced phosphorylation of two serines (Ser 32, Ser 36) on the IκBα molecule.[24,25] To determine the inhibitory effect of PDTC on NF-κB activity correlated with IκB degradation, the cytoplasmic p-IκBα level of OD-treated peritoneal macrophages was examined by Western blotting using a specific antibody to p-IκBα. The results indicated that pretreatment with PDTC prevented IκBα phosphorylation after 60-min incubation with rIFN-γ plus OD (Fig. 5).

Direct Effect of OD on Cancer Cells To test whether OD could affect the growth of cancer cells, we evaluated cell viability by MTT assay in HepG2 cells and HT-29 cells. OD (1 mg/ml) showed a direct effect in the HepG2 cells but no effect in the HT-29 cells (Fig. 6).

Characterization of the Principal Components of OD Finally, we standardized OD by nuclear magnetic resonance (NMR) spectrometry.[26—28] The water extract of OD was analyzed by ^{1}H-NMR spectroscopy of D_2O solubles. It gave the main biological compound of which the signals were clearly visible in the ^{1}H-NMR spectrum of the extract. Especially obvious were chemical shifts between 3.20 and 3.95 ppm and 4.65 (d) and 5.25 (d) ppm, indicative of a glucoside group and between 0.80 and 2.80 ppm. ^{1}H-NMR analysis of the water extract showed that it contained glucoside as the main component with a steroid (Fig. 7).

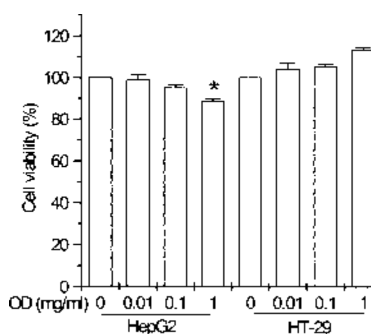

Fig. 6. Effects of OD on Cancer Cells

Cell viability was evaluated by MTT colorimetric assay 24 h after OD treatment (0.01—1 mg/ml) in HepG2 or HT-29 cells. The percentage of viable cells was over 98% in each condition. Data represent the mean$\pm$S.E.M. of three independent experiments. $*p<0.01$ compared with control value.

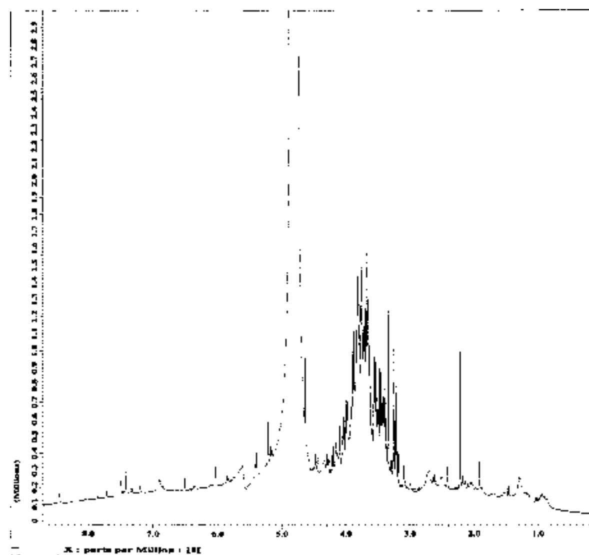

Fig. 7. ^{1}H-NMR Spectrum of OD

^{1}H-NMR study was carried out using a Varian Unity 500 (500 MHz, Japan) spectrometer at 25 °C. Acquisition parameters are: content, single-pulse experiment; spec site, JNM-ECP500; spec type, DELTA-NMR; data format, 1D complex; dimensions, X; dim title, 1H; dim size, 16384; dim units, [ppm].

DISCUSSION

In this study, we demonstrated that NO production in mouse peritoneal macrophages by OD could be highly stimulated in combination with rIFN-γ. OD had a maximal effect on NO production at a concentration of 1 mg/ml in rIFN-γ treated mouse peritoneal macrophages. The results of this study suggest that OD may provide a second signal for synergistic induction of NO production in mouse peritoneal macrophages. *N*MMA, an analogue of L-arginine, inhibited rIFN-γ- plus OD-induced NO production in peritoneal macrophages. The effective concentration of *N*MMA for NO inhibition without any toxicity on cells is up to 10 mM, which is the concentration commonly used. The strong inhibition of nitrite production by *N*MMA indicates that it is likely to depend upon NOS.

At present, the precise physiological significance of NO production by OD is unknown. However, an important role for the synthesis of NO in host defense against pathogens and tumor cells has been recognized.[7] NO generation by

iNOS also influences the cytotoxicity of macrophages and tumor-induced immunosuppression. NO produced by OD indicates that it may provide various activities such as anti-microbial, antitumoral, and antiviral activity under specific conditions *in vivo*. In addition, since NO has emerged as an important intracellular and intercellular regulatory molecule with functions as diverse as vasodilation, neural communication, cell growth regulation, and host defense,[29] it is tempting to hypothesize that this molecule is involved in the local control of various fundamental processes.

NO synthesis was greatly diminished by neutralizing antibody for TNF-α.[30] Our results also suggest that the synergism between IFN-γ and OD in increasing NO synthesis by stimulated macrophages could be due to TNF-γ secretion triggered by OD in IFN-γ-primed macrophages. It has previously been reported that LPS stimulation of rIFN-γ-primed macrophages induces NF-κB activation.[31] NF-κB is now known to be ubiquitously expressed and to play a major role in controlling the expression of proteins involved in immune, inflammatory, and acute-phase responses.[32] Expression of iNOS and TNF-α genes is dependent on the activation of NF-κB.[33] We found that the addition of the NF-κB inhibitor PDTC inhibits the synergistic effect of OD with rIFN-γ on NO and TNF-α production. It also inhibits the phosphorylation of IκB in OD-plus rIFN-γ-stimulated macrophages. These results suggest that OD increases NO and TNF-α production through NF-κB activation. The NF-κB system may provide a future target of anticancer therapy.

In conclusion, our results demonstrate that OD acts as an accelerator of peritoneal macrophage activation by rIFN-γ *via* a process involving L-arginine-dependent NO production and that it increases the production of TNF-α significantly *via* NF-κB activation. Although the precise mechanism by which OD promotes NO and TNF-α production induced by rIFN-γ remains to be elucidated, NO and TNF-α production by OD might explain its beneficial effect in the treatment of tumors.

The amounts of OD used in this study were high concentrations, raising the possibility that the active agent or agents in the OD represent a small component of the total mass. Therefore further investigation is necessary to clarify unknown constituents that may be more active than OD itself. Studies on the isolation and characterization of the active chemical constituents are in progress.

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