Red Ginseng Acidic Polysaccharide (RGAP) in Combination with IFN-γ Results in Enhanced Macrophage Function through Activation of the NF-κB Pathway

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This study examined the effects of red ginseng acidic polysaccharide (RGAP) on macrophage-mediated cytotoxicity towards murine melanoma B16 cells. RGAP alone had no effect on killing of tumor cells. RGAP treatment increased the production of interleukin-1 (IL-1), IL-6, and nitric oxide (NO) by macrophages, whereas tumor necrosis factor (TNF-α) and reactive oxygen species (ROS) production were not changed by RGAP. However, treatment of macrophages with a combination of RGAP and recombinant interferon-γ (rIFN-γ) enhanced killing of tumor cells. In addition, the combination treatment showed marked cooperative induction of IL-1, IL-6, TNF-α, and NO production. Electrophoretic mobility shift assay analysis revealed that treatment of macrophages with RGAP plus rIFN-γ induced the activation of the nuclear factor-kappa B (NF-κB) transcription factor. In agreement with this, the combination treatment resulted in increased NF-κB-p65 expression. The present results demonstrate synergistic effects on macrophage function of RGAP in combination with rIFN-γ, and suggest that NF-κB plays an important role in mediating these effects. These data also support the development of clinical studies of this combination.

Key words: red ginseng acidic polysaccharide; macrophage; cytokines; NO; NF-κB

The root of Panax ginseng C.A. Meyer (Araliaceae) is one of the most popular natural tonics which used in Asian countries. Water extracts of ginseng have shown antitumor activity in some kinds of tumor cells in mice1) and have inhibited the incidence of lung tumors induced by a wide range of carcinogens.2) In epidemiologic studies, ginseng intake reduced the incidence of human cancer.3) However, the active substance and its mechanism of action remain unclear. In a previous study, we isolated red ginseng acidic polysaccharide (RGAP) from Korean red ginseng and found it to induce a proliferation of spleen cells, to decrease the antibody-forming cell response to sheep red blood cells and to stimulate nitric oxide production in murine peritoneal macrophages in vivo.4,5) In addition, RGAP has recently been found to show immunomodulating and anticancer properties in a murine-transplanted tumor cell model.6) Macrophages have been found to be important components of host defenses against bacterial infections and murine tumor cells (lymphoma and mastocytoma).7) In addition, macrophages can be activated to kill tumor cells in vitro by a nonspecific, extracellular mechanism that might be important in host defenses against neoplastic cells in vivo.9) Peritoneal macrophages can be stimulated by a variety of agents, such as IFN-γ, lipopolysaccharides, and other microbial products,10–14) and some of these have also been shown to trigger the release of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, and nitrite, and to induce tumorcidal activity by the macrophages.15–17) Furthermore, cytokines such as TNF-α, IL-1, and IFN-γ have been found to modulate macrophage functions by...
producing synergistic effects with other agents, inducing the release of cytotoxic molecules from macrophages.8,18,19)

A large number of studies have shown that different signaling pathways participate in the activation of macrophages by various stimuli.20–23) In addition, previous studies have demonstrated that the action of several transcription factors, including the NF-κB, mediate the activation of human monocytes.24,25) NF-κB was found to be an inducible transcription factor, required for the transcription of TNF-α, iNOS, IL-6, and cyclo-oxygenase-2.25)

In the present study, we examined the effects of RGAP on macrophage-mediated cytotoxicity against B16 tumor cells, and attempted to determine whether this effect is dependent on NF-κB activation.

Materials and Methods

Mice, chemicals, and reagents. C57BL/6 male mice (6–8 weeks old, 17–21 g) were obtained from Charles River Breeding Laboratories (Atsugi, Japan). Unless otherwise indicated, all chemicals were purchased from Sigma Chemical (St. Louis, MO). RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY). 6-amino-4-(4-phenoxyphenylethylamino) quinazoline (quinazoline) and N\(^{G}\)G-monomethyl-L-arginine (NMMA) were obtained from Calbiochem (LaJolla, CA). IL-1, IL-6, and TNF-α ELISA kits were purchased from R&D System (Minneapolis, MN). Recombinant mouse IFN-γ was obtained from R&D System. Antibodies against p65 and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All tissue culture reagents as well as the thioglycollate broth were assayed for any endotoxin contamination by the Limulus lysate test (E-Toxate kit, Sigma), and the level of endotoxin was found to be < 0.006 EU (endotoxin units).

Isolation of inflammatory peritoneal macrophages. Thioglycollate-elicited peritoneal exudate cells were obtained from C57BL/6 male mice after they were given an intraperitoneal injection of 1 ml of Brewer. Thioglycollate broth (4.05 g/100 ml) (Difco Laboratories, Detroit, MI) was followed by a lavage of the peritoneal cavity with 5 ml of medium 3–4 d later. The cells were washed twice and resuspended in RPMI-1640 (GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 μg/ml) (RPMI-FBS). The macrophages were isolated from the peritoneal exudate cells as described previously.22) The peritoneal exudate cells were seeded on teflon-coated petri dishes (100 × 15 mm) at densities of 5–6 × 10⁵ cells/cm², and the macrophages were allowed to adhere for 2–3 h at 37°C in a 5% CO₂ humidified atmosphere. Teflon-coated petri dishes were prepared by spraying them with aerosolized teflon (Fisher Scientific, Pittsburgh, PA), followed by sterilization using ultraviolet light for 3 h. The non-adherent cells were removed by washing the dishes twice with 10 ml of prewarmed medium and incubating them for 10 min at 4°C. The supernatants were then carefully removed and discarded, and the plates were washed once with a prewarmed Dulbecco’s Phosphate Buffered Saline solution (PBS) (GIBCO). Cold PBS (15 ml) containing 1.5% FBS was then added, followed by the addition of 0.3 ml of 0.1 M EDTA (pH 7.0). The plates were incubated for 15 min at room temperature, and the macrophages were removed by rinsing the plates 10 times using a 10-ml syringe. The viability of the detached cells was assessed by trypan blue exclusion and the proportion of macrophages was determined after cytoplasmic staining with acridine orange by fluorescence microscopy. More than 95% of the cell preparations were viable, and they contained > 95% macrophages.

Preparation of red ginseng acidic polysaccharide. Red ginseng made by steaming and drying fresh root of Panax ginseng C.A. Mayer was cut to mill. Powdered red ginseng was percolated with 5 volumes of 85% ethanol to extract off ethanol-soluble materials. The remaining residues were percolated with 5 volumes of distilled water, and the water-soluble extracts were concentrated with a vacuum evaporator. The concentrate was dialyzed against running tap water for 7 d to completely cut off small molecules of less than 15 kDa. Four volumes of absolute ethanol were added to precipitate the polysaccharide in the inner dialysate. The precipitate was dried in a vacuum drying oven, and was finally used as a red ginseng acidic polysaccharide (RGAP). The chemical composition of RGAP was 56.9% acidic sugars and 28.3% neutral sugars as determined by carbazole assay and phenol-sulfuric assay respectively.26) The protein content of RGAP was below 0.1% as determined by the Lowry method. Less than 0.006 EU (endotoxin units) of endotoxin was present in 1 mg of RGAP as tested by Limulus amebocyte lysate assay. This level of endotoxin did not affect the experimental results obtained by RGAP.

Determination of phagocytosis. Phagocytic activity was measured using the assay system described previously.27) Macrophages were treated with indicated concentrations of RGAP for 18 h, and then washed with RPMI1640 to remove RGAP. Cells were incubated with 5 × 10⁶ particles of zymosan and 600 μg/ml of nitroblue tetrazolium. After 1 h of incubation, plates were centrifuged at 4°C to stop ingestion of zymosan, and supernatant was removed by flipping. The optical density of the reduction product of NBT, a purple insoluble formazan, was determined at 540 nm using a Molecular device microplate reader (Molecular device, Menlo Park, CA). It was not necessary to solubilize the formazan before taking the measurement of absorbance.
Macrophage-mediated cytotoxicity. The assay for macrophage cytotoxicity was based on an assay described elsewhere. Briefly, the macrophages (1 x 10^5 cells/well) were plated into 96-well microtiter plates and incubated in various concentrations of RGAP or a combination of RGAP and rIFN-γ for 18 h at 37°C in a 5% CO₂ incubator. The macrophages were washed with RPMI-FBS to remove the stimulants and co-incubated with B16 melanoma cells (ATCC, Rockville, MD) (1 x 10^4/wells; initial effector:target cell ratio, 10:1) at 37°C in a 5% CO₂ incubator. The cell density was then assessed by incubating the cells with 25 μg/ml of MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide] for a further 4 h. The formazan produced was dissolved in dimethyl sulfoxide, and the optical density of each well was determined at a wavelength of 540 nm using a Molecular device microplate reader. Cytolytic activity was expressed as the percentage tumor cytotoxicity, as follows:

% Cytotoxicity
= \left\{1 - \text{O.D. of \{target + macrophages\}} \right\}/\text{O.D. of target (nontreated)} \right\} \times 100

Nitrite determination. Peritoneal macrophages were cultured with RGAP, rIFN-γ, or a combination of RGAP and rIFN-γ for 18 h. The amount of NO₂⁻ accumulated in the culture supernatants was measured as described previously. Briefly, 100 μl of the supernatant was removed from each well and placed into an empty 96-well plate. After adding 100 μl of Griess reagent to each well, the absorbance was measured at 550 nm using a Molecular Device microplate reader. NO₂⁻ concentration was calculated from a NaNO₂ standard curve. The NO₂⁻ levels are indicative of the amount of NO production. The Griess reagent was prepared by mixing 1 part of 0.1% naphthylethylene diamine dihydrochloride in distilled water with 1 part of 1% sulfanilamide in 5% concentrated H₃PO₄.

Determination of intracellular reactive oxygen species (ROS). Peritoneal macrophages were seeded at a density of 1 x 10⁶ cells in 60-mm culture dishes and cells treated with RGAP, rIFN-γ, or RGAP plus rIFN-γ for 18 h. Cells were stained at 37°C for 15 min with 5 μM CM-H₂DCFDA (Molecular Probes, Eugene, OR), an ROS sensitive dye, for ROS measurement. The intracellular ROS level was measured by flow cytometry (Win BRYTE HS, Bio-RAD, Santa Cruz, CA). Data were expressed as percentages of untreated control from triplicate samples, and at least 10,000 cells were analyzed in each experiment.

Cytokine determination by ELISA. Peritoneal macrophages were cultured with RGAP, rIFN-γ, or a combination of RGAP and rIFN-γ for 18 h at 37°C in a 5% CO₂ incubator. The culture supernatants were collected, and TNF-α, IL-1, and IL-6 concentrations in the culture supernatants were determined using a DuoSet Elisa kit (R&D Systems). The manufacturer’s instructions were followed. Samples were assessed in triplicate relative to standards supplied by the manufacturer.

Preparation of nuclear extract and electrophoretic mobility shift assay (EMSA). The macrophages (2 x 10⁵ cells/ml) were suspended in RPMI 1640 medium supplemented with 10% FBS and placed in 6-well plates (3 ml/well) and incubated at 37°C. The cells were then incubated for 6 h after first exposing them to RGAP, rIFN-γ, or a combination of RGAP and rIFN-γ, and were collected on ice before isolation of the nuclear extracts. They were washed with ice-cold phosphate-buffered saline and suspended in 200 μl of a lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and 1 mM dithiothreitol). They were then allowed to swell on ice for 15 min, after which 12.5 μl of 10% nonidet P-40 was added. The tube was mixed thoroughly for 10 s using a Vortex mixer prior to centrifugation (10,000 x g) at 4°C for 3 min. The nuclear pellets obtained were resuspended in 25 μl of an ice-cold nuclear extraction buffer (20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA and 1 mM dithiothreitol), and kept on ice for 15 min with intermittent agitation. The samples were subjected to centrifugation for 5 min at 4°C, and the supernatant was stored at −70°C. An aliquot was taken and the protein concentration was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). EMSAs were carried out using a digoxigenin (DIG) gel shift kit (Boehringer Mannheim Biochimica, Mannheim, Germany) according to the manufacturer’s protocol. Briefly, oligonucleotide 5’-AGTTGAGGGGACTTTCCAGG-3’ containing the NF-kB binding site was DIG-labeled using a 3’-end labeling kit, and the DNA probe was incubated with 10 μg of the nuclear extract at room temperature for 10 min. Subsequently, the protein-DNA complexes were separated on a 6% polyacrylamide gel and electrically transferred to a nylon membrane (Boehringer Mannheim Biochimica) for chemiluminescence band-detection. The specificity of the binding was examined using competition experiments, where a 100-fold excess of the unlabeled oligonucleotide with the same sequence or unrelated oligonucleotide (5’-CTAGTGACCTAAGGCCGATC-3’) was added to the reaction mixture before addition of the DIG-labeled oligonucleotide.

Western blot analysis. Western blot analysis was performed by a modification of a technique described elsewhere. After treatment, the cells were washed twice in PBS and suspended in a lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% NP40, 100 μg/ml, phenylsulfonyl fluoride, 2 μg/ml, aprotinin, 1 μg/ml, pepstatin, and 10 μg/ml, leupeptin). The cells were placed on ice for 30 min. The supernatant was collected
after centrifugation at 15,000 × g for 20 min at 40 °C. Nuclear extracts were also prepared as above to determine the levels of NF-κB protein p65. The protein concentration was determined using a Bio-Rad protein assay (Bio-Rad Lab.) with BSA as the standard. The whole lysates (20 μg) were resolved on a 7.5% SDS–polyacrylamide gel, transferred to an immobilon polyvinylidene difuride membrane (Amersham, Arlington Heights, IL), and probed with the appropriate antibodies. Blots were then developed using an enhanced chemiluminescence (ECL) kit (Amersham). In all immunoblotting experiments, the blots were reprobed with anti-β-actin antibody as a control for protein loading.

**Statistical analysis.** Each result is reported as means ± S.E.M. Two-way analysis of variance was used for analysis of differences among groups, and significant values are represented by an asterisk. (*p < 0.05).

**Results**

As a preliminary experiment to determine the effects of RGAP on murine peritoneal macrophages, the phagocytic activity of RGAP-treated macrophages was tested. As shown in Fig. 1, phagocytic activity increased slightly. This increase was statistically significant at p < 0.05. This observation led to the question whether RGAP affects tumoricidal activity of murine peritoneal macrophages against B16 tumor cells, which were used as targets since they are either TNF-α or NO sensitive. Thiglycollate-elicited macrophages were treated with various concentrations of RGAP for 18 h and cocultured with the B16 tumor cells. RGAP did not induce tumoricidal activity in mouse peritoneal macrophages (Fig. 2A). However, RGAP in combination with rIFN-γ synergistically increased tumoricidal activity as compared to rIFN-γ alone (Fig. 2B). RGAP and rIFN-γ did not exert any cytotoxic activity on B16 cells by themselves. Although the data shown represent an initial effector:target cell ratio of 10:1, similar results were obtained at higher effector:target cell ratios (data not shown).

In the next set of experiments, we determined the secretory effects of RGAP on the production of TNF-α, IL-1, and IL-6 by macrophages. Peritoneal macrophages were treated with various concentrations of RGAP for

**Fig. 1.** Effects of RGAP on Phagocytosis of Murine Peritoneal Macrophages.

Macrophages were treated with various concentrations of RGAP for 18 h. They were then incubated with 5 × 10⁶ particles of zymosan and 600 μg/ml of NBT. Phagocytosis was measured as OD 540 nm. The results shown are the mean ± S.E.M for three independent experiments. *p < 0.05, significantly different from control (no treatment).

**Fig. 2.** Cytotoxicity of B16 Tumor Cells by RGAP-(A) and rIFN-γ Plus RGAP (B)-Treated Peritoneal Macrophages.

A, Macrophages were stimulated with indicated concentrations of RGAP for 18 h. B, Macrophages were treated with RGAP (1 μg/ml), rIFN-γ (50 U/ml), or rIFN-γ (50 U/ml) plus RGAP (1 μg/ml) for 18 h. The tumoricidal activity of macrophages was determined as described in “Materials and Methods.” The data shown are the results of an initial effector/target ratio of 10:1. The results are reported as mean ± S.E.M for three independent experiments. As a positive control, rIFN-γ (50 U/ml) combined with LPS (1 μg/ml) was used. *p < 0.05, significantly different from the control (no treatment). **p < 0.05, significantly different from the group treated with rIFN-γ and with RGAP alone.
Augmented Macrophage Functions by RGAP and IFN-γ

Fig. 3. Enhanced TNF-α, IL-1 and IL-6 Production by Peritoneal Macrophages Cultured with rIFN-γ Plus RGAP.

Macrophages were treated with RGAP (1 μg/ml), rIFN-γ (50 U/ml), or rIFN-γ (50 U/ml) plus RGAP (1 μg/ml) for 18 h. Culture supernatants were collected, and the levels of TNF-α, IL-1, and IL-6 were measured by ELISA. The results shown are the mean ± S.E.M for three independent experiments. *p < 0.05, significantly different from control (no treatment). †p < 0.05, significantly different from the group treated with rIFN-γ and with RGAP alone.

Fig. 4. Nitrite (A) and ROS (B) Production from the Peritoneal Macrophages Stimulated with rIFN-γ Plus RGAP.

Macrophages were treated with RGAP (1 μg/ml), rIFN-γ (50 U/ml), or rIFN-γ (50 U/ml) plus RGAP (1 μg/ml) for 18 h. A, The culture supernatants were collected and the nitrite level was measured by the Griess method. B, Macrophages were incubated with CM-H2DCFDA (5 μM) for 15 min at 37°C for the ROS level. Stained cells were analyzed by flow cytometry. The results are mean ± S.E.M of triplicates from one representative experiment, and at least 10,000 cells were analyzed in each experiment. *p < 0.05, significantly different from control (no treatment). †p < 0.05, significantly different from the group treated with rIFN-γ and with RGAP alone.

18 h and culture supernatants were assayed for cytokines. As shown in Fig. 3, lower production of IL-1 and IL-6 was observed with 1 μg/ml of RGAP as compared to that of medium-treated control, while production of TNF-α was not altered by treatment with RGAP. We also determined whether nitric oxide (NO) and reactive oxygen species (ROS) was produced by peritoneal macrophage in treatment with RGAP (Fig. 4). The supernatants in a well plate exposed to RGAP for 18 h...
were measured by the Griess method. ROS was determined by staining RGAP-treated cells with CM-H2-DCFDA (5 μM). RGAP did not affect the increase in NO and ROS production at 1 mg/ml. In contrast, the combination of RGAP and rIFN-γ showed a synergistic effect on the secretion of cytokines and NO production but not on ROS as compared to RGAP or rIFN-γ alone (Figs. 3 and 4).

Since IFN-γ is known to serve a macrophage-priming function, delayed addition of RGAP was also examined. Sequential addition of RGAP to rIFN-γ-containing cultures had an effect similar to that of simultaneous addition of rIFN-γ and RGAP at the time cultures were initiated (Table 1). TNF-α and NO production was increased after sequential RGAP addition to rIFN-γ.

It is well documented that NF-κB is a ubiquitous transcription factor that plays a crucial role in macrophage activation via the transcription of inflammatory mediators downstream of multiple types of stimulatory events. In addition, NF-κB regulates inflammatory gene expression in cases such as those of cytokines and NO in many immune effector cells, including macrophages.30,31 Therefore, the ability of RGAP in combination with rIFN-γ to activate NF-κB was tested in an attempt to identify the nuclear factors that contribute to the activation of macrophages. An electrophoretic mobility shift assay showed that the combination treatment produced a marked increase in NF-κB DNA binding activity in macrophages as compared with a single agent (Fig. 5A). Since the p65 subunit of NF-κB has been demonstrated to exert critical activity in the transcription of many inflammatory genes, we also performed a western blot assay to detect expression of p65 NF-κB protein. As shown in Fig. 5B, stimulation of macrophages with this combination resulted in increased protein synthesis of p65 NF-κB.

Among the effectors produced by activated macrophages, NO is an important mediator, responsible for cytostatic and/or cytolytic activity of macrophages both in vitro and in vivo. Since RGAP with rIFN-γ stimulated NO production and NF-κB activation, it was of interest to determine whether NF-κB activation is involved in increase in NO production induced by the combination treatment in macrophages. Pretreatment with NF-κB inhibitor (quinazoline, 10 μM) 1 h prior to RGAP plus rIFN-γ exposure caused a significant attenuation of iNOS-NO system activity (Fig. 6). These results suggest that NF-κB activation is involved in the increase in iNOS-NO system activity induced by the combination treatment.

**Discussion**

In the present study, we identified the augmented effects of RGAP plus rIFN-γ on murine peritoneal macrophages. Our initial experiments investigating the effects of RGAP on cellular and secretory activities showed an enhanced phagocytic effect on macrophages. In addition, only low levels of IL-1 and IL-6 were detectable in RGAP-treated macrophages, but RGAP synergistically induced tumoricidal activity, cytokines, and NO production by macrophages when the cells were treated by rIFN-γ.

Studies have recently demonstrated that NO production increased in macrophages from RGAP-treated mice.3 This observation is in contrast with present results showing that RGAP did not induce NO production. This discrepancy possibly arose from differences in cellular microenvironments found in vitro and in vivo. In

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### Table 1. Enhanced TNF-α and NO Production after Sequential RGAP Addition of IFN-γ

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<th>Untreated</th>
<th>IFN-γ</th>
<th>RGAP</th>
<th>IFN-γ + RGAP</th>
</tr>
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<tbody>
<tr>
<td>TNF-α (pg/ml)</td>
<td>25 ± 2</td>
<td>35 ± 5</td>
<td>27 ± 2</td>
<td>164 ± 9*</td>
</tr>
<tr>
<td>Nitrite (μM)</td>
<td>2.2 ± 0.1</td>
<td>2.5 ± 0.2</td>
<td>2.3 ± 0.1</td>
<td>5.9 ± 0.2*</td>
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*Control medium (no treatment) or IFN-γ (50 U/ml)-containing medium was added to cells at initiation of the cultures. Six hours later, RGAP (1 μg/ml) was added. Supernatants were harvested for TNF-α and NO measurement 18 h after that. Results are mean ± S.E.M of triplicate samples.

* p < 0.05, significantly different from control (no treatment).
fact, natural killer-, T and B-cell-derived products can act on macrophages to enhance their responses.

It is known that treatment of resident macrophages with IFN-γ induces a primed state. Primed macrophages not only have phagocytic activity, but become fully activated cytolytic when stimulated with LPS. These cytolytic macrophages might be able to secrete various cytokines, such as TNF-α, IL-1, and IL-6. Secretion of these mediators was found to require a triggering signal such as LPS, providing some evidence to the role of priming and triggering signals in inducing the complete cytolytic function. In this study, sequential treatment with rIFN-γ and RGAP yielded clear evidence of activation of murine peritoneal macrophages, resulting in increased production of TNF-α and NO. These data indicate that RGAP provides a second signal for synergistic induction of secretory activities in mouse peritoneal macrophages. In addition, the results reported here suggest that there is no significant difference in the type or pattern of macrophage function when combined treatment occurs with simultaneous or sequential agent addition.

MHC compatibility between effector macrophages and target cells might be important in the recognition process leading to macrophage cytotoxicity. Our results indicate that tumoricidal activities of macrophages from syngeneic C57/BL6 mice against B16 melanoma cell were significantly increased by treatment of RGAP in combination with rIFN-γ. In addition, RGAP plus IFN-γ-activated macrophages from allogeneic CD-1 mice is cytotoxic to B16 and to P815 target cells (data not shown). Based on these findings, it is plausible that macrophage-mediated cytotoxicity induced by the combination might be MHC-unrestricted and not directly dependent on the recognition of tumor-specific antigen, expressed by the tumor cells.

Activation of transcription factors is critical in the regulation of multiple genes involved in the control of immune and inflammatory responses. Among various transcription factors, NF-κB regulates many important functions in macrophages and plays a crucial role in expression of cytokines and NO production. NF-κB is present in the cytosol as a pre-formed trimeric complex, and the P50/P65 protein dimer is associated with an inhibitory protein known as IκB. NF-κB p65 heterodimers migrate to the nucleus upon activation, where NF-κB binds to its consensus sequence on the promoter–enhancer region of various genes and regulates transcription of specific genes. Hence we investigated the DNA binding activity of NF-κB and the levels of nuclear NF-κB P65 protein. It was found that treatment with RGAP in combination with rIFN-γ

Fig. 6. Inhibition by the NF-κB Inhibitor, Quinazoline of iNOS-NO System Activity in rIFN-γ (50 U/ml) Plus RGAP (1 μg/ml)-Stimulated Macrophages.
A, Nitrite levels. B, Western blot analysis of iNOS protein levels. Data are expressed as mean ± SEM of triplicate independent experiments. *p < 0.05, as compared with control (no treatment).
increased the DNA binding activity of NF-κB and increased amounts of NF-κB P65 protein in nuclear extracts of macrophages treated with the combination in comparison with both those treated with a single agent and untreated control.

Furthermore, our results indicated that the increase in iNOS-NO system activity induced by the combination of two agents was mediated by activation of NF-κB at transcriptional levels. Pretreatment with an NF-κB inhibitor, quinazoline, inhibits the synergistic effect of RGAP with rIFN-γ on NO production, implying that the combination of two agents increases NO production through NF-κB activation.

In addition to binding sites for NF-κB, the promoter-enhancer region of iNOS gene contains binding sites for several other transcription factors such as AP-1, interferon response element, and the STAT family of transcription factors. Hence our data do not totally rule out the possibility that other transcription factors are involved in the combination-induced enhancement of iNOS-NO system activity in macrophages.

The data reported here suggest, therefore, that the combination of RGAP plus IFN-γ provides potential antitumor therapeutic efficacy via induction of macrophage activation with concomitant cytokine induction and induction of NO. In addition, the present results suggest that NF-κB is involved in macrophage secretory and cellular activities induced by the combination treatment. Further studies are needed to clarify how this modulation occurs and to what extent it occurs in vivo.

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