FEBS Letters 509 (2001) 156–160 FEBS 25548

Ursolic acid enhances nitric oxide and tumor necrosis factor-α production via nuclear factor-κB activation in the resting macrophages

Ho Jin You[a,c], Chul Yung Choi[b], Ji Young Kim[b], Sung Jun Park[c], Kyung-Soo Hahm[c], Hye Gwang Jeong[a,b,c,*]

[a] *Department of Pharmacy, College of Pharmacy, Chosun University, 375 Soesuk-dong, Kwangju 501-759, South Korea*
[b] *Department of Pharmacology, School of Medicine, Chosun University, Kwangju, South Korea*
[c] *Research Center for Proteineous Materials, Chosun University, Kwangju, South Korea*

Received 4 September 2001; revised 29 October 2001; accepted 6 November 2001

First published online 19 November 2001

Edited by Masayuki Miyasaka

Abstract Ursolic acid (UA), a pentacyclic triterpene acid, is reported to have anti-tumor activities; however, the mechanism underlying its anti-tumorigenic effects is poorly understood. To further determine the mechanism of UA, we investigated the effects of UA on the release of nitric oxide (NO) and tumor necrosis factor-α (TNF-α), and on the level of inducible nitric oxide synthase (iNOS) and TNF-α gene expression in mouse resting macrophages. We found that UA elicited a dose-dependent increase in NO and TNF-α production, and the level of iNOS and TNF-α mRNA. Transient expression and electrophoretic mobility shift assays with nuclear factor-κB (NF-κB) binding sites revealed that the increased level of iNOS mRNA and TNF-α mRNA induced by UA were mediated by the NF-κB transcription factor complex. These results demonstrate that UA stimulates NO and TNF-α release and is able to upregulate iNOS and TNF-α expression through NF-κB transactivation in the resting macrophages.

Key words: Ursolic acid; Macrophage; Inducible nitric oxide synthase; Tumor necrosis factor-α; Nuclear factor-κB

1. Introduction

Macrophages play a significant role in host defense mechanisms. When activated, they inhibit the growth of a wide variety of tumor cells and microorganisms. Nitric oxide (NO), a free-radical gas, is synthesized by inducible nitric oxide synthase (iNOS) [1,2] and mediates diverse functions, including vasodilatation, neurotransmission, the inhibition of platelet aggregation, immunoresponses, and the inhibition of extracellular matrix production [3]. NO has been identified as the major effector molecule involved in the destruction of tumor cells by activated macrophages [4,5]. Moreover, the involvement of NO during non-specific host defense, macrophage-mediated killing, and the inhibition of the proliferation of microorganisms and tumor cells both in vitro and in vivo have been previously demonstrated [6,7]. Such NO-mediated tumoricidal activity is induced by DNA damage and leads to apoptotic cell death [8]. The administration of NOS inhibitors to mice has been found to promote the growth of several transplantable tumors [7], and melanoma cells transfected with iNOS cDNA were found not proliferate and metastasize well [9]. Tumor necrosis factor-α (TNF-α) is produced by activated macrophages, fibroblasts, and many different types of cells. TNF-α has also been recognized and well characterized as an important host defense molecule that affects tumor cells [4,10–12]. In macrophages, nuclear factor κB (NF-κB) in cooperation with other transcription factors was found to coordinate the expression of genes encoding iNOS. Moreover, NF-κB plays a critical role in the activation of immune cells by upregulating the expression of many cytokines essential for immune response [13].

Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical diseases, with relatively little knowledge of their modes of action. There is a growing interest in the elucidation of the biological roles of triterpenoid compounds, the major components of some traditional medicinal plants [14,15], in terms of hepatoprotectory, analgesic, anti-tumor, anti-inflammatory and immunomodulatory effects. Ursolic acid (UA; 3β-hydroxy-urs-12-en-28-oic acid), is a triterpenoid compound, which is present in many kinds of medicinal plants, such as *Eriobotrya japonica, Rosmarinus officinalis*, and *Glechoma hederaceae* [16–18], in the form of free acid or as aglycones of triterpenoid saponins [14]. It has been reported that UA produce a wide variety of anti-tumor activity, including inhibition of tumorigenesis [18] and tumor promotion [16,17], and the induction of tumor cell differentiation [19], an anti-angiogenic effect [20], and anti-invasive activity in human fibrosarcoma cells [21]. UA also induces growth inhibition at the G1 phase of the cell cycle and apoptosis in certain cancer cell systems [22,23]. However, the mechanisms by which UA induces such cellular effects are poorly understood. To further characterize the mechanisms involved in UA-mediated anti-tumor effects, we tested the hypothesis that UA derives its anti-tumor effect by the release of NO from macrophages. We investigated the effects of UA on the release of NO and TNF-α, and on the level of iNOS and TNF-α gene expression in mouse macrophages.

2. Materials and methods

2.1. Chemicals

Chemicals and cell culture materials were obtained from the following sources: UA, *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) and Polymyxin B sulfate (Sigma); MTT-based colorimetric assay kit (Roche); LipofectAMINE Plus, RPMI 1640, fetal bovine serum, and

*Corresponding author. Fax: (82)-62-230 6639.
E-mail address: hgjeong@mail.chosun.ac.kr (H.G. Jeong).

PII: S0014-5793(01)03161-1

penicillin–streptomycin solution (Life Technologies); pGL3-4KB-Luc and the luciferase assay system (Promega); pCMV-β-gal (Clonetech); other chemicals were of the highest commercial grade available.

2.2. Animals

Specific pathogen free-BALB/C mice (female, 5–7 weeks old) were obtained from KRIBB (South Korea). Mice were housed under normal laboratory conditions, i.e. at 21–24°C and 40–60% relative humidity, under a 12 h light/dark cycle with free access to standard rodent food and water.

2.3. Preparation of peritoneal macrophages and cell cultures

Peritoneal macrophages were isolated from mice and cultured as described previously [24]. RAW 264.7 cells, mouse macrophage cell line, were obtained from the American Type Culture Collection (Bethesda, MD, USA), and grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 humidified incubator. UA was dissolved in dimethylsulfoxide and added directly to the culture media. Control cells were treated only with solvents, the final concentration of which never exceeded 0.1% and this concentration did not show any effect on the assay systems.

2.4. Cell viability

Cell viability was assessed using a MTT-based colorimetric assay kit (Roche), according to the manufacturer's instructions.

2.5. Nitrite assay

Peritoneal macrophages (2×10^5 cells/ml) or RAW 264.7 cells (5×10^5 cells/ml) were cultured in 48-well plates. After incubating for 24 h, NO synthesis was determined by assaying the culture supernatants for nitrite, the stable reaction product of NO with molecular oxygen, using Ellman's reagent as described previously [24].

2.6. Immunoassay of TNF-α

Peritoneal macrophages or RAW 264.7 cells were cultured at a density of 2×10^6 cells/ml for 6 h in 24-well plates. TNF-α production was quantified by sandwich immunoassays as described previously [24]. Recombinant murine TNF-α was used as a standard.

2.7. Endotoxin Assay

An E-Toxate test (Limulus Amebocyte Lysate; Sigma) was used to assay UA for the presence of Gram-negative bacterial endotoxin (LPS), according to the manufacturer's instructions.

2.8. RNA preparation and iNOS mRNA analysis by reverse transcription-polymerase chain reaction (RT-PCR)

RAW 264.7 cells were cultured with UA at a density of 1×10^6 cells/ml for 6 h. Total cellular RNA was isolated by the acidic phenol extraction procedure of Chomczynski and Sacchi [25]. cDNA synthesis, semiquantitative RT-PCR for iNOS, TNF-α, and β-actin mRNA, and the analysis of results were performed as described previously [24]. cDNA was synthesized from 2 μg of total RNA using an Omniscript RT-PCR kit as instructed. A cycle number that fell within the exponential range of response for iNOS (754 bp, 35 cycles), TNF-α (692 bp, 35 cycles), and β-actin (153 bp, 17 cycles) was used. PCR reactions were electrophoresed through a 2.5% agarose gel and visualized by ethidium bromide staining and UV irradiation. Gel images were captured on a Gel Doc Image Analysis System (Kodak), and the yield of PCR products was normalized to β-actin after quantitative estimation using NIH Image software (Bethesda, MD, USA). The relative expression levels were arbitrarily set at 1.0 in the control group.

2.9. Transfection and luciferase and β-galactosidase assays

RAW 264.7 cells (5×10^5 cells/ml) were plated in each well of a 12-well plate, and 12 h later, transiently co-transfected with the plasmids pGL3-4κB-Luc and pCMV-β-gal using LipofectAMINE Plus according to the manufacturer's protocol. Briefly, the transfection mixture containing 0.5 μg of pGL3-4κB-Luc and 0.2 μg of pCMV-β-gal was mixed with the LipofectAMINE Plus reagent and added to cells. After 18 h, cells were treated with UA or LPS for 12 h, and then lysed. Luciferase and β-galactosidase activity were determined as described previously [24]. Luciferase activity was normalized using β-galactosidase activity and was expressed relative to the control activity.

2.10. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as previously described [26]. Two double-stranded deoxyoligonucleotides containing the NF-κB binding site (5′-GGGGACTTTCC-3′) [2] were end-labeled with [γ-^{32}P]dATP. Nuclear extracts (5 μg) were incubated with 2 μg of poly (dI-dC) and the ^{32}P-labeled DNA probe in binding buffer (100 mM NaCl, 30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml concentration each of aprotinin and leupeptin) for 10 min on ice. DNA binding was separated from the free probe using a 4.8% polyacrylamide gel in 0.5×TBE buffer (44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA). Following electrophoresis, the gel was dried and subjected to autoradiography.

2.11. Statistical analysis

All experiments were repeated at least three times. The Student's *t*-test was used to assess the statistical significance of differences. A confidence level of <0.05 was considered significant.

3. Results and discussion

Since UA is known to have anti-tumor activity [16–23], we investigated the effects of UA on the release of NO and TNF-α, and its effects on the level of iNOS and TNF-α gene expression in mouse macrophages. Our findings indicate that in macrophages, UA stimulates NO and TNF-α release and is able to upregulate iNOS and TNF-α expression through NF-κB transactivation. UA-induced NO production was assessed using the Griess reaction, and the basal level of NO in untreated peritoneal macrophages was found to be less than 2 μM (Table 1). Moreover, UA showed a significant effect on nitrite production from 1 μM. Upon UA stimulation, nitrite release by peritoneal macrophages increased in a dose-dependent manner in the range of 1–10 μM (Table 1), and showed a cytotoxic action toward macrophages at concentrations over 30 μM (data not shown). In addition, the potent macrophage activator LPS increased nitrite synthesis

Table 1
Effects of UA on NO and TNF-α secretion

Treatment[a]	Macrophages		RAW 264.7	
	Nitrite (μM)[b]	TNF-α (ng/ml)[c]	Nitrite (μM)[b]	TNF-α (ng/ml)[c]
Control	1.61 ± 0.21	0.65 ± 0.11	2.15 ± 0.33	0.73 ± 0.13
UA 1 μM	3.35 ± 0.43*	2.11 ± 0.32*	6.01 ± 6.43*	2.82 ± 0.41*
UA 5 μM	7.65 ± 0.83*	5.21 ± 0.63*	22.42 ± 3.17*	6.51 ± 0.82*
UA 10 μM	12.11 ± 1.36*	9.33 ± 1.23*	43.91 ± 5.21*	11.05 ± 1.65*
LPS 0.5 μg/ml	13.52 ± 1.44*	11.56 ± 1.31*	52.31 ± 6.13*	14.52 ± 1.64*

Values are expressed as mean ± S.D. of three individual experiments, performed in triplicate. *$P<0.05$, significantly different from the control
[a]Murine peritoneal macrophages (2×10^5 cells/ml for nitrite assay, and 2×10^6 cells/ml for TNF-α immunoassay) or RAW 264.7 cells (5×10^5 cells/ml for nitrite assay, and 2×10^6 cells/ml for TNF-α immunoassay) were cultured with the indicated concentrations of UA or LPS.
[b]Supernatants were harvested 24 h later and assayed for NO.
[c]Supernatants were harvested 6 h later and assayed for TNF-α.

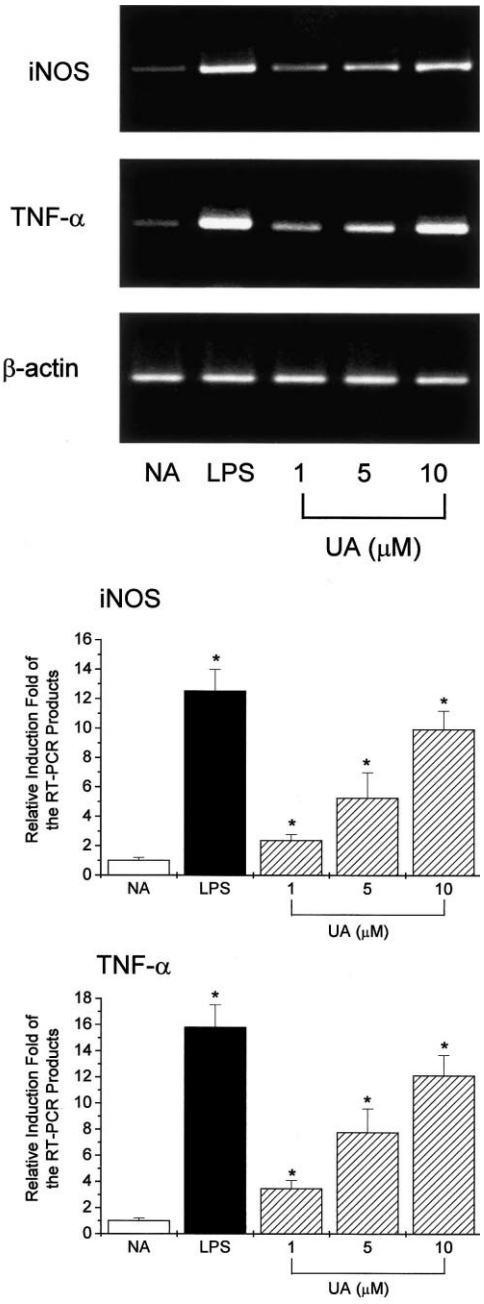

Fig. 1. Effects of UA on iNOS and TNF-α mRNA expression. RAW 264.7 cells (1×10^6 cells/ml) were cultured for 6 h in the presence of media alone, with the indicated concentrations of UA, or with LPS (0.5 μg/ml). Cells were lysed and total RNA was prepared for the RT-PCR analysis of gene expression. PCR amplification of the housekeeping gene, β-actin, was performed for each sample. The PCR amplification products were electrophoresed in 2.5% agarose gel and stained with ethidium bromide. One of three representative experiments is shown. The ratio of the RT-PCR products of iNOS or TNF-α to β-actin was calculated. Induction-fold is represented as a mean ± S.D. of three separate experiments. $^*P<0.05$, significantly different from the control.

compared to the control. Consistent with these findings, UA also induced nitrite generation in a dose-dependent manner in RAW 264.7 cells (Table 1). As found in the NO assay, upon UA stimulation, TNF-α secretions increased in a dose-dependent manner in peritoneal macrophages and RAW 264.7 cells (Table 1). These results suggest that the secretion of NO and

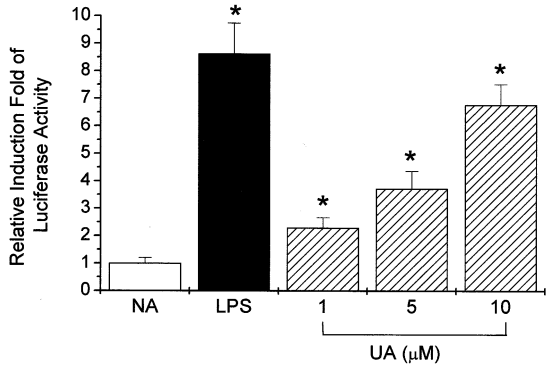

Fig. 2. Effects of UA on NF-κB-dependent luciferase gene expression. RAW 264.7 cells (5×10^5 cells/ml) were transiently co-transfected with pGL3-4κB-Luc and pCMV-β-gal. After 18 h, cells were treated with the indicated concentrations of UA or LPS (0.5 μg/ml) for 12 h. Cells were then harvested, and luciferase and β-galactosidase activities determined. Luciferase activities were expressed relative to the control. Each bar shows the mean ± S.D. of three independent experiments, performed in triplicate. $^*P<0.05$, significantly different from the control.

TNF-α is regulated by the same mechanism, or that TNF-α, which is produced first, induces NO secretion via an autocrine or paracrine system. TNF-α is the first compound of the TNF-α and NO series to be secreted by macrophages [27]. Thus, TNF-α is involved in the early phase of the cytokine cascade and induces NO production.

NO is involved in the killing and proliferative inhibition of microorganisms, the destruction of tumor cells by activated macrophages, and is a component of the non-specific host defense [5–9,28,29]. Furthermore, it has demonstrated that murine macrophages stimulated by TNF-α [5] produce NO via the expression of the iNOS gene, and it is believed that the reactive nitrogen intermediates so induced play a significant role in tumoricidal and microbiocidal activities [5]. TNF-α, an endogenous factor with tumor-selective cytotoxicity, has been recognized as an important host defense molecule that affects tumor cells. Moreover, the induction of NO and TNF-α production and gene expression by activated macrophages can lead to cytostatic and cytotoxic effects on malignant cells [4,6,10–12,24]. Because of the pivotal role of NO and TNF-α in the anti-microbial and tumoricidal activities of macrophages, significant effort has been focused on developing therapeutic agents that regulate NO and TNF-α

Table 2
Effects of polymyxin B on NO and TNF-α secretion by UA and LPS

Treatment[a]	Nitrite (μM)[b]	TNF-α (ng/ml)[c]
Control	2.17 ± 0.32*	0.67 ± 0.12*
UA	47.31 ± 5.61	10.05 ± 1.34
UA+polymyxin B	48.95 ± 5.37	11.51 ± 1.46
LPS	55.74 ± 6.12	13.52 ± 1.85
LPS+polymyxin B	14.82 ± 1.72*	5.37 ± 0.07*

$^*P<0.05$, significantly different from the LPS and UA
[a]RAW 264.7 cells (5×10^5 cells/ml for nitrite assay, and 2×10^6 cells/ml for TNF-α immunoassay) cultured with UA (10 μM) or LPS (0.5 μg/ml), in the presence or absence of polymyxin B (10 μg/ml).
[b]Supernatants were harvested 24 h later and assayed for NO.
[c]Supernatants were harvested 6 h later and assayed for TNF-α. Values are expressed as mean ± S.D. of three individual experiments, performed in triplicate.

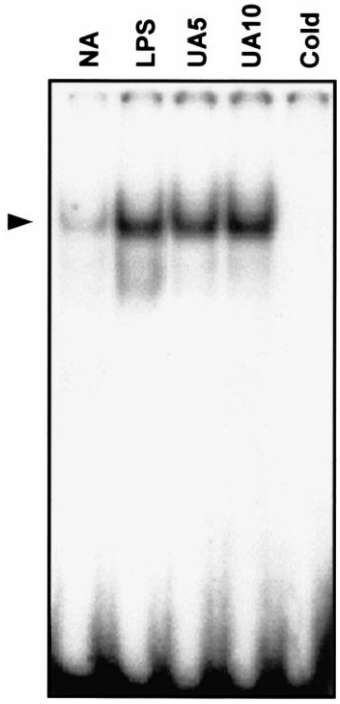

Fig. 3. Effects of UA on NF-κB-binding. RAW 264.7 cells were treated with LPS (0.5 μg/ml) or UA for 1 h. Nuclear extracts were isolated and used in an EMSA with ^{32}P-labeled NF-κB oligonucleotide as a probe, as described in Section 2. The arrow indicates the NF-κB binding complex. Cold: 200-fold molar excess of non-labeled NF-κB probe. One of three representative experiments is shown.

production [30]. Based on these results and the relationship between nitrite and the cytolytic function of macrophages against a variety of tumors [7,28], we suggest that the antitumor effect of UA might be mediated in part through the activation of NO and TNF-α secretion.

Macrophages can be induced to produce NO and TNF-α by LPS, endotoxins, or cytokines [28]. To confirm that the ability of UA to induce NO and TNF-α could not be attributed to LPS contamination, the UA was tested for the presence of contaminating LPS using the *Limulus* amoebocyte lysate test. The level of LPS in UA was below the detection limit, which was less than 12.5 pg/ml (data not shown). Polymyxin B sulfate has been used previously as a LPS inhibitor in macrophage cultures [31], and although UA contained no detectable activity in the *Limulus* amoebocyte lysate assay, we checked for possible LPS contamination in UA, by adding polymyxin B to cell cultures treated with UA. As shown in Table 2, polymyxin B effectively inhibited the NO and TNF-α secretion induced by LPS, but had no effect on the UA. This result demonstrated that the production of NO and TNF-α by UA was unlikely to have resulted from LPS contamination in the UA.

As stated above, UA induced macrophage secretion of NO and TNF-α. In order to determine whether UA regulates NO and TNF-α secretion at the mRNA level, a RT-PCR assay was conducted. LPS was used as a positive control. Consistent with the results obtained from the NO assay, iNOS mRNA levels were markedly increased by UA treatment (Fig. 1). This result indicates that UA upregulated, in a dose-dependent manner, NO accumulation in macrophages. Therefore, we believe that increased NO production by UA is regulated through transcriptional activation. Under the same treatment conditions, the TNF-α gene expression marker of macrophage activation was also examined, and similarly, UA was found to significantly enhance the expression levels of the TNF-α gene (Fig. 1). This result is consisted with that obtained from the immunoassay of TNF-α in macrophages, and indicates that the UA also upregulates TNF-α accumulation in a dose-dependent manner.

NF-κB is a member of the Rel family and is a common regulatory element in the promoter region of many cytokines. In activated macrophages, NF-κB in synergy with other transcriptional activators plays a central role in coordinating the expression of genes encoding iNOS, TNF-α, and interleukin-1 (IL)-1 [13]. To further investigate the role of UA on iNOS and TNF-α gene expression, the effect of UA on NF-κB-dependent gene expression was assessed using the luciferase reporter gene assay. RAW 264.7 cells were transiently transfected with a plasmid containing four copies of the NF-κB binding sites and the luciferase activities were measured. LPS, an immunostimulatory agent, was used as a positive control. Nearly a six-fold increase in the luciferase activity was observed compared to the unstimulated control cells when cells were stimulated with LPS. Consistent with NO production and iNOS mRNA measurement, UA also significantly increased NF-κB-dependent luciferase activities in a dose-dependent manner (Fig. 2). To further investigate the putative mechanism by which UA activates iNOS, the effects of UA on the activation of a family of transcription factors was monitored by gel shift assay. NF-κB binding activity was examined in light of its critical role in the regulation of iNOS and TNF-α. EMSA demonstrated that UA induced a marked increase in NF-κB binding to its conserved site that could be visualized by a distinct band (Fig. 3). These results indicate that the upregulation of the iNOS and TNF-α gene by UA is mediated by the transactivation of NF-κB. Although we demonstrated the upregulatory ability of UA on iNOS and TNF-α expression in macrophages, the mechanism by which UA stimulated iNOS and TNF-α expression in macrophages remains unknown, such as the activation of Raf-1 and MAP kinases [32]. Additional studies are needed to answer these questions and to elucidate the mechanisms involved.

Previously, several studies have shown that the treatment of UA or synthetic oleananes leads to reduction of LPS- or PMA(phorbol 12-Myristate 13-acetate)-inducible NO production, and iNOS and inducible cyclooxygenase expression through inhibition of NF-κB activation [33,34]. We also confirmed that UA decreased LPS-inducible NO production and iNOS expression (data not shown). In the present study, however, we found that the treatment of UA in the absence of LPS caused enhancement of NO and TNF-α production, as well as iNOS and TNF-α mRNA induction via transactivation of NF-κB. These results have provided evidence that UA may have dual effects on the iNOS expression depending on the resting or stimulating state of macrophages. UA is able to induce the basal (intrinsic) level of NO and TNF-α production even though UA cause the reduction of LPS-inducible NO production and iNOS expression. iNOS-derived NO has been found to inhibit the DNA binding activities of cysteine-rich transcription factors through reactive nitrogen oxide species [35]. For example, NO inhibits the DNA binding activity of NF-κB, AP-1, *c*-Myb, SP1, and EGR-1 [35–39]. Each of these contains a cysteine residue in or near their DNA binding

region, which probably represents the target site for *S*-nitrosation. Therefore, there is a possibility that the blocking of LPS-induced NF-κB activation by UA treatment [33] might be mediated in part through the inhibition of DNA binding activity of NF-κB by UA-induced NO. The biological significance of difference effects of UA on the NO production between the basal and LPS-inducible state need to be determined.

The cytotoxicity of tumor cells is dependent on the activation of macrophages, which is strongly correlated with the expression patterns of several cytokine mediators. Marcinkiewicz et al. [40] demonstrated that the increasing NO levels enhance the release of TNF-α and reactive oxygen species. This effect may be due to both an increased generation of the superoxide anion, and the preferential formation of peroxynitrite, which can be formed by direct reaction between NO and the superoxide radical; both have powerful cytotoxic properties [41]. The decomposition product of peroxynitrite and the hydroxy radical is believed to be the most toxic oxygen molecule in vivo [42]. NO and TNF-α were investigated in the current study to confirm the possibility that UA might be an immunostimulator, and UA was found to elicit NO and TNF-α production. This result supports the possibility that NO and TNF-α induction by UA may contribute in vivo to its immunomodulatory and anti-tumoricidal activities. Biological response modifiers are widely used in cancer immunochemotherapy to potentiate therapeutic efficacy or to alleviate the toxicity of cytotoxic anti-cancer agents. It is interesting that UA can upregulate iNOS expression in macrophages. NO is known to act as a powerful inducer of apoptosis through upregulation of the proapoptotic proteins, such as P53 and Bax [35]. Thus, the effect of UA on the cell cycle and apoptosis in cancer cells [22,23] may be mediated in enhanced NO production. The use of UA has been recommended for skin cancer therapy in Japan [43]. Further studies on UA will be needed to prove its clinical usefulness in cancer therapy and its effectiveness in other diseases. In order to investigate the overall anti-tumor effect of UA, a study on the in vivo induction of gene expression and the production of immunomodulatory cytokines in mice is underway in our laboratory. However, the exact mechanism underlying UA-induced NO production and release remains to be elucidated.

Acknowledgements: This study was supported by a grant from the Ministry of Science and Technology, Korea and the KOSEF through the Research Center for Proteineous Materials.

References

[1] Xie, O.-W., Cho, H.J., Calaycay, J., Mumford, R.A. and Swiderek, K.M. (1992) Science 256, 225–228.
[2] Baeuerle, P.A. and Henkel, T. (1994) Annu. Rev. Immunol. 12, 141–179.
[3] Nijkamp, F. and Folkerts, G. (1997) Clin. Exp. Allergy 27, 347–350.
[4] Duerksen-Hughes, P.J., Day, D., Laster, S.M., Zachariades, N.A., Aquino, L. and Gooding, L.R. (1992) J. Immunol. 149, 2114–2122.
[5] Lorsbach, R.B., Murphy, W.J., Lowenstein, C.J., Snyder, S.H. and Russell, S.W. (1993) J. Biol. Chem. 268, 1908–1913.
[6] Stuehr, D.J. and Nathan, C.F. (1989) J. Exp. Med. 169, 1543–1555.
[7] Farias-Eisner, R., Sherman, M.P., Aerberhard, E. and Chaudhuri, G. (1994) Proc. Natl. Acad. Sci. USA 91, 9407–9411.
[8] Nicotera, P., Brune, P. and Bagetta, G. (1997) Trends Pharmacol. Sci. 18, 189–190.
[9] Xie, K., Huang, S., Dongm, Z., Juang, S.-H. and Gutman, M. (1995) J. Exp. Med. 181, 1333–1343.
[10] Balkwill, F.R., Maylor, M.S. and Malik, S. (1990) Eur. J. Cancer 26, 641–644.
[11] Gorelik, L., Bar-Dagan, Y. and Mokyr, M.B. (1996) J. Immunol. 156, 4298–4308.
[12] Lasek, W., Feleszko, W., Golab, J., Stoklosa, T. and Marczak, M. (1997) Cancer Immunol. Immunother. 45, 100–108.
[13] Ghosh, S., May, M.J. and Kopp, E.B. (1998) Annu. Rev. Immunol. 16, 225–260.
[14] Mahato, S.B., Sarkar, S.K. and Poddar, G. (1988) Phytochemistry 27, 3037–3067.
[15] Liu, J. (1995) J. Ethnopharmacol. 49, 57–68.
[16] Tokuda, H., Ohigashi, H., Koshimizu, K. and Ito, Y. (1986) Cancer Lett. 33, 279–285.
[17] Ohigashi, H., Takamura, H., Koshimizu, K., Tokuda, H. and Ito, Y. (1986) Cancer Lett. 30, 143–151.
[18] Huang, M.T., Ho, C.T., Wang, Z.Y., Ferraro, T., Lou, Y.R., Stauber, K., Ma, W., Georgiadis, C., Laskin, J.D. and Conney, A.H. (1994) Cancer Res. 54, 701–708.
[19] Lee, H.Y., Chung, H.Y., Kim, K.H., Lee, J.J. and Kim, K.W. (1994) J. Cancer Res. Clin. Oncol. 120, 513–518.
[20] Sohn, K.H., Lee, H.Y., Chung, H.Y., Young, H.S., Yi, S.Y. and Kim, K.W. (1995) Cancer Lett. 94, 213–218.
[21] Cha, H.J., Bae, S.K., Lee, H.Y., Lee, O.H., Sato, H., Seiki, M., Park, B.C. and Kim, K.W. (1996) Cancer Res. 56, 2281–2284.
[22] Es-Saady, D., Simon, A., Jayat-Vignoles, C., Chulia, A.J. and Delage, C. (1996) Anticancer Res. 16, 481–486.
[23] Baek, J.H., Lee, Y.S., Kang, C.M., Kim, J.A., Kwon, K.S., Son, H.C. and Kim, K.W. (1997) Int. J. Cancer 73, 725–728.
[24] Choi, C.Y., Kim, J.Y., Kim, Y.S., Chung, Y.C., Hahm, K.-S. and Jeong, H.G. (2001) Cancer Lett. 166, 17–25.
[25] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[26] Jeon, Y.J., Lee, J.S. and Jeong, H.G. (1999) Chem. Biol. Interact. 118, 113–125.
[27] Martin, J.H. and Edwards, S.W. (1993) J. Immunol. 150, 3478–3486.
[28] Stuehr, D.J. and Marletta, M.A. (1985) Proc. Natl. Acad. Sci. USA 82, 7738–7742.
[29] Hibbs, J.B., Taintor, R.R. and Vavrin, Z. (1987) Science 235, 473–476.
[30] Poderoso, J.J., Carreras, M.C., Schopfer, F., Lisdero, C.L., Riobo, N.A., Giulivi, C., Boveris, A.D., Boveris, A. and Cadenas, E. (1999) Free Radical. Biol. Med. 16, 925–935.
[31] Manthey, C.L., Brandes, M.E., Perera, P.U. and Vogel, S.N. (1992) J. Immunol. 149, 2459–2465.
[32] Meyer, C.F., Wang, X., Chang, C., Templeton, D. and Tan, T.H. (1996) J. Biol. Chem. 271, 8971–8976.
[33] Suh, N., Honda, T., Finlay, H.J., Barchowsky, A., Williams, C., Benoit, N.E., Xie, Q.W., Nathan, C., Gribble, G.W. and Sporn, M.B. (1998) Cancer Res. 58, 717–723.
[34] Subbaramaiah, K., Michaluart, P., Sporn, M.B. and Dannenberg, A.J. (2000) Cancer Res. 60, 2399–2404.
[35] Kröncke, K.D., Fehsel, K., Suschek, C. and Kolb-Bachofen, V. (2001) Int. Immunopharmacol. 1, 1407–1420.
[36] Matthews, J.R., Botting, C.H., Panico, M., Morris, H.R. and Hay, R.T. (1996) Nucleic Acids Res. 24, 2236–2242.
[37] Moormann, A.M., Koenig, R.J. and Meshnik, S.R. (1996) Redox. Rep. 2, 249–256.
[38] Nikitovic, D., Holmgren, A. and Spyrou, G. (1998) Biochem. Biophys. Res. Commun. 242, 109–112.
[39] Brendeford, E.M., Andersson, K.B. and Gabrielsen, O.S. (1998) FEBS Lett. 425, 52–56.
[40] Marcinkiewicz, J., Grabowska, A. and Chain, B. (1995) Eur. J. Immunol. 25, 947–951.
[41] Klebanoff, S.J. (1993) Free Radical Biol. Med. 14, 351–360.
[42] Yamauchi, N., Kuriyama, H., Watanabe, N., Neda, H., Maeda, M. and Niitsu, Y. (1989) Cancer Res. 49, 1671–1675.
[43] Muto, Y., Ninomiya, M. and Fujiki, H. (1990) Jpn. J. Clin. Oncol. 20, 219–224.