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Vitamin C Blocks TNF-α-induced NF-*k*B Activation and ICAM-1 Expression in Human Neuroblastoma Cells

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Interactions of the cell adhesion molecules are known to play important roles in mediating inflammation. The proinflammatory cytokine, tumor necrosis factor-α (TNF-α), activates the NF-*k*B signaling pathway, which induces the expression of various genes, such as intercellular adhesion molecule-1 (ICAM-1). In this study, the effect of vitamin C on the ICAM-1 expression induced by TNF-α in a human neuroblastoma cell line, SK-N-SH was investigated. Treatment with vitamin C resulted in the downregulation of the TNF-α-induced surface expression and ICAM-1 mRNA levels in a concentration-dependent manner. Moreover, a gel shift analysis indicated that vitamin C dose-dependently inhibited the NF-*k*B activation and I*k*Bα degradation induced by TNF-α. Taken together, these results suggest that vitamin C downregulates TNF-α-induced ICAM-1 expression via the inhibition of NF-*k*B activation.

Key words: Vitamin C, ICAM-1, TNF-α, NF-*k*B

INTRODUCTION

Cell adhesion plays a crucial role in the immune system, and is an important factor in inflammatory neurological disorders. During neurological diseases many factors have been detected in the central nervous system microenvironment. Proinflammatory cytokines appear to be released by infiltrating leukocytes, by endothelial cells and resident glial cells, astrocytes and microglia (Owens *et al.*, 1994; Schobitz *et al.*, 1994). The local release of multiple cytokines by infiltrating leukocytes and/or CNS cells contributes to the pathogenesis of inflammatory neurological disease. In the CNS, TNF-α has been shown to upregulate intercellular adhesion molecule-I (ICAM-1) and vascular cell adhesion molecule-I (VCAM-1) on neural cells (Birdsall, 1991; Birdsall *et al.*, 1992). Elevated levels of ICAM-1 expression have been shown to be critically involved in the development of various diseases (Davies *et al.*, 1993; Szekanecz *et al.*, 1994). In addition, ICAM-1 expression by tumor cells has been reported to be a major contributing factor facilitating metastatic progression (Johnson, 1991).

The induction of ICAM-1 depends on the transcription factor, nuclear factor *k*B (NF-*k*B), which is activated by proinflammatory cytokines, such as IL-1 and TNF-α (Baeuerle and Henkel, 1994). NF-*k*B exists in a latent form in the cytoplasm of unstimulated cells, complexed to an inhibitor protein from the I*k*B family. Several stimuli activate NF-*k*B by augmenting the activity of the I*k*B kinase (IKK) complex, which phosphorylates I*k*B at sites that trigger their ubiquitination and proteasome-mediated degradation (Finco and Baldwin, 1995). Many NF-*k*B-regulated genes, including adhesion molecules and monocyte chemoattractant protein-1 (MCP-1), and several cytokines, are important mediators of inflammation (Baeuerle and Henkel, 1994).

Although the mechanisms used by external oxidants in order to regulate cell growth are largely unknown, it is possible that they modulate cell signalling and/or the activity of some transcription factors. Among those transcription factors, NF-*k*B is one of the more extensively studied. Several supplementation studies have recently reported the clear benefit of antioxidant intervention on the mortality from an established disease (Boaz *et al.*, 2000). However, the probability of a benefit from antioxidant intervention may be influenced by many factors, including

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the degree of primary pathology and low plasma ascorbate (Khaw *et al.*, 2001)

Vitamin C is an essential vitamin for humans, primates, guinea pigs, and few other animals and insects that lack the enzyme, L-gulono-lactone oxidase, the final enzyme in the biosynthetic pathway for vitamin C (Nishikimi and Yagi, 1991; Nishikimi *et al.*, 1994). Vitamin C is transported into most cells in the oxidized form, dehydrovitamin C acid (DHA), via facilitative glucose transporters (Vera *et al.*, 1993; Rumsey *et al.*, 2000), and as ascorbic acid in specialized cells, by sodium-dependent ascorbic acid transporters (Tsukaguchi *et al.*, 1999). When transported as DHA, vitamin C is rapidly reduced inside the cell and accumulates as ascorbic acid (Vera *et al.*, 1995). Vitamin C is a strong antioxidant that sustains a balance of the reactive oxygen species (ROS) generated in the course of aerobic ATP generations (Frei *et al.*, 1989; Strain and Benzie, 1999) inhibits cell death and prevents mutations induced by ROS (Guaiquil *et al.*, 2001; Lutsenko *et al.*, 2002). Additionally, there have been studies pointing to the role of vitamin C in an enhanced host defense and in the modulation of inflammatory reactions (Heuser *et al.*, 1997; Williams *et al.*, 1984). Although there has been much interest in the effect of vitamin C on the pathways to NF-*k*B and inflammatory responses, its effect on the intracellular signaling pathways by which TNF-α causes ICAM-1 expression has not been elucidated in neuronal cells.

This study tested whether vitamin C would alter the TNF-α-induced expression of ICAM-1 in human neuroblastoma cells. Follow-up studies have determined the effects of vitamin C on the activation of NF-B caused by TNF-α treatment.

MATERIALS AND METHODS

Reagents

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St Louis, USA). The anti-ICAM-1 (BBA3) and anti-IκBα antibodies were purchased from Santa Cruz Biotechnology (USA). The anti-mouse IgG-HRP and *p*-nitrophenyl phosphate were purchased from Sigma Chemical Co. and the fetal bovine serum (FBS) was purchased from Gibco, USA.

Cell culture

The neuroblastoma cell line, SK-N-SH cells, was purchased from the American Type Culture Collection (ATCC; Manassas, VA). Cells were maintained in RPMI 1640 and 10% fetal bovine serum at 37°C in a humidified atmosphere, consisting of 5% CO_2 and 95% air.

Cytotoxicity evaluation

Vitamin C and TNF-α, at the concentrations presented herein, were shown to be non-toxic by the following procedure. The viability of neuroblastoma cells treated with the test compound and TNF-α, respectively, was determined using the MTT assay. Cells were cultured in gelatin coated 96-well microplates (Costar Products, Cambridge, MA) until confluent. Cells were treated with or without the test compounds in quadruplicate for the indicated times, and subsequently 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide (MTT) was added for 4 h. In addition, a visual inspection of the cells was performed by trypan blue exclusion staining under an inverted microscope.

ELISA for measurement of adhesion molecules

The cell surface expression of the adhesion molecules on the endothelial monolayers was quantified using ELISA, but with a modification to the methods described previously (Son *et al.*, 2001). Cells were seeded at a concentration of 2×10^4 cells/well in 96-well, flat bottomed gelatin-coated plates (Nalgen Nunc International, IL). Cells were pretreated with various doses of vitamin C (1-10 mM) before stimulation with TNF-α for 1 h. After vitamin C incubation, the cells were washed with phosphate buffered saline, pH 7.4 (PBS), and incubated with TNF-α (5 ng/mL) for the times indicated in the text. Cells were washed with PBS, and then fixed with 10% glutaraldehyde for 30 min at 4°C. Bovine serum albumin (1.0% in PBS) was added to the cells in order to reduce the non-specific binding. Cells were incubated with anti-ICAM-1 monoclonal antibodies or the isotype matched control antibodies (0.25 g/mL, diluted in blocking buffer) overnight at 4°C, washed with PBS, followed by incubation with the alkaline phosphatase-conjugated goat anti-mouse secondary antibodies (1 g/mL, diluted in PBS). Cells were then washed with PBS and exposed to the peroxidase substrate (*p*-nitrophenyl phosphate 1 mg/mL in 0.1 M glycine buffer, pH 10.4, containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$). The absorbance was determined at 405 nm using a Molecular device microplate reader (Menlo Park, CA). The absorbance values of the isotype matched control antibodies were taken as the blank, and were subtracted from the experimental values.

Measurement of mRNA levels by reverse transcription polymerase chain reaction (RT-PCR)

After treatment, total cellular RNA was isolated by the guanidinium lysis method, as described by Chomczynski & Sacchi (1987). The yield and purity of the RNA were confirmed by measuring the ratio of the absorbencies at 260 and 280 nm. To identify ICAM-1-specific cDNA, PCR was performed using ICAM-1-specific primers. ICAM-1-specific primers were synthesized: sense primer, 5'-CTG-CTGGGAATTTTCTGGCCAC-3' and antisense primer, 5'-

CTATGGCAACGACTCCTTCTCG-3'. The β-actin PCR primers were 5'-GGAGAAGAGCTACGAGCTGC-3' (sense) and 5'-CCGGACTCGTCATACTCCTG-3' (antisense). Each primer pair was chosen to span the introns of their respective human genes. Using these primers, it is expected that DNA fragments of 313 and 238 bp would be amplified from the ICAM-1 and β-actin RNA, respectively.

Preparation of nuclear extract and electrophoretic mobility shift assay (EMSA)

SK-N-SH cells (2×10^6 cells/mL), suspended in RPMI 1640 medium supplemented with 10% FBS, were placed in 6-well plates (3 mL/well) and incubated at 37°C in the presence of vitamin C (1-10 mM). After 1 h, each well was treated with TNF-α (5 ng/mL) for 1 h. The cells were collected on ice before isolation of the nuclear extracts. The cells (2×10^6 cells) were washed with ice-cold phosphate-buffered saline (PBS) and suspended in 200 μL of lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and 1 mM dithiothreitol). The cells were allowed to swell on ice for 15 min, after which 12.5 μL of 10% nonidet P-40 was added. The tube was then mixed thoroughly using a Vortex mixer for 10 sec prior to centrifugation (10.000 × g) at 4°C for 3 min. The nuclear pellets thus obtained were resuspended in 25 μL of 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 under intermittent agitation. The samples were subjected to centrifugation for 5 min at 4, and the supernatant stored at -70°C. An aliquot was taken and its protein concentration determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). EMSAs were carried out using a digoxigenin (DIG) gel shift kit (Boehringer Mannheim Biochemica, Mannheim, Germany), according to the manufacturer's instructions. Briefly, the oligonucleotide, 5'-AGTTGAGGGGACTTTCCCAGGC-3', containing a *k*B-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. The protein-DNA complexes were then separated on a 6% polyacrylamide gel, and electrically transferred to a nylon membrane (Boehringer Mannheim Biochemica) for chemiluminescence band-detection. The specificity of binding was examined by competition experiments, where a 100-fold excess of unlabeled oligonucleotide with the same sequence or unrelated oligonucleotide (5'-CTAGTGAGC-CTAAGGCCGGATC-3') was added to the reaction mixture prior to the addition of the DIG-labeled oligonucleotide.

Western Blotting for I*k*Bα

After treatment, the cells were washed twice in PBS and suspended in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% NP40, 100 g/mL phenylsulfonyl fluoride, 2 g/mL aprotinin, 1 g/mL pepstatin and 10 g/mL leupeptin), and placed on ice for 30 min. After centrifugation at 15000 × g for 20 min at 4°C, the supernatant was collected and the cytosol fraction obtained. Its protein concentration was measured using the above-mentioned kit, and then stored at -70°C. An aliquot of the cytosol fraction containing 100 μg was resolved by 10% SDS-polyacrylamide gel, transferred to an immobilon polyvinylidene difuride membrane (Amersham, Arlington heights, IL) and probed with the anti-I*k*Bα antibody (Santa Cruz Biotechnology) for 1 h. The blot was developed using the enhanced chemiluminescence (ECL) kit (Amersham). The blot was also reprobed with an anti-β-actin antibody for the control of the protein loading.

RESULTS

Effect of vitamin C on TNF-α-induced ICAM-1 expression in neuroblastoma cells

It has previously been shown that some cytokines induce the expression of adhesion molecules on neuronal cells and facilitate the adherence of leukocytes (Birdsall, *et al.*, 1992). To establish if TNF-α induced the expression of ICAM-1 on neuroblastoma cells, the levels of ICAM-1 on the surface of these cells was examined. As shown in Fig. 1, the ICAM-1 expression was constitutively expressed on SK-N-SH cells, and the treatment of SK-N-SH cells with TNF-α increased the expression of ICAM-1.

The effect of vitamin C on the TNF-α stimulated

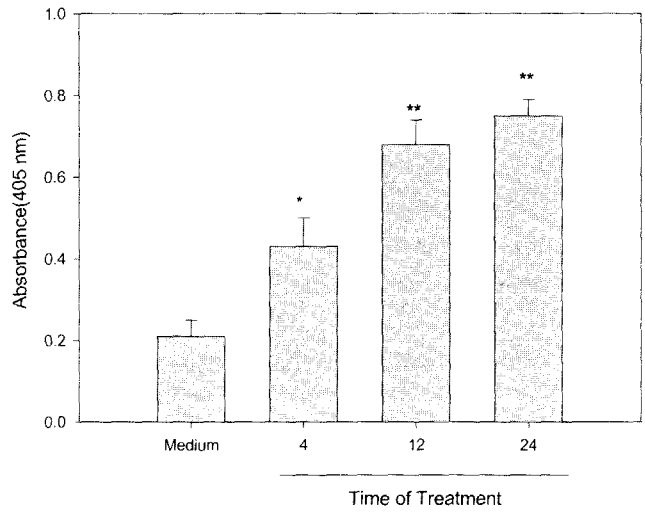

Fig. 1. Expression of ICAM-1 on SK-N-SH cells induced by TNF-α. The neuroblastoma cells were stimulated with TNF-α (5 ng/mL) for various times, and expression of ICAM-1 measured by ELISA, as described in the Methods. Each experiment was performed in quadruplicate. The results are the mean ± S.E.M from three independent experiments. *p<0.05, **p<0.01; significantly different from control (no treatment).

upregulation of ICAM-1 was examined in neuroblastoma cells, which was determined on the same 96-well plate. The incubation time and concentration of this compound used in these experiments had no effect on the viability, as determined by trypan blue staining. Morphologically, the cell monolayers of endothelial cells appeared normal, with no detachment of the cells observable after exposure to the doses of vitamin C used (data not shown). These concentrations were in complete agreement with the optimum concentrations of the compound needed in the other reported system (Strain *et al.*, 1999; Bowie and O'Neill, 2000). As detected by ELISA, ICAM-1 was expressed at low levels on unstimulated cells, but was induced about 4-fold by TNF-α stimulation at 12 h. Vitamin C treatment resulted in a reduction of the TNF-α-induced ICAM-1 expression, in a dose-dependent manner (Fig. 2A).

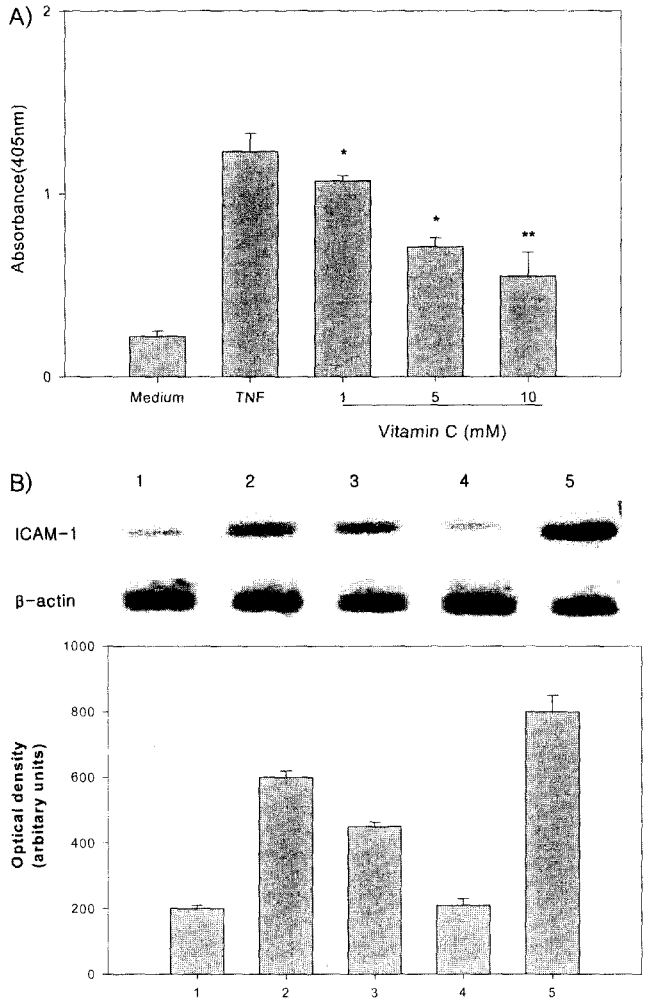

Fig. 2. Dose dependent inhibition of TNF-α-induced ICAM-1 expression by vitamin C. (A) Three independent experiments were performed in which the confluent cells were pretreated with vitamin C (1, 5 and 10 mM) for 1 h before stimulation with TNF-α (5 ng/mL) for 12 h. The cell surface ICAM-1 expressions were measured by ELISA. The results are the mean ± S.E.M from three independent experiments. *$p<0.05$, **$p<0.01$; significantly different from TNF-α treated cells. (B) The confluent cells were pretreated with vitamin C (1, 5 and 10 mM) for 1 h before stimulation with TNF-α (5 ng/mL) for 4 h. The levels of the mRNA for ICAM-1 were determined by RT-PCR. β-actin served as a housekeeping gene. A typical result from three independent experiments is shown. Relative changes in the ICAM-1 mRNA bands were measured using densitometric analysis and optical densities (n=3), which are plotted in arbitrary units as the means S.E.M. Unstimulated (lane 1); TNF-α + Vitamin C (lanes 2-4); TNF-α-stimulated (lane 5).

The experiments described above demonstrated that vitamin C markedly inhibits the cell surface expression of ICAM-1 in cells stimulated with TNF-α. It was possible that the interference of vitamin C with TNF-α-induced ICAM-1 upregulation also occurs at the transcriptional level. To address this possibility, and measure the reproducibility of the ELISA analysis, total cellular RNAs were isolated from cells and analyzed by RT-PCR. Cells were pretreated with various doses of vitamin C for 1 h, and then stimulated with TNF-α for 4 h. Treatment with vitamin C markedly decreased the induction of ICAM-1 mRNAs (Fig. 2B). The level of mRNA inhibition appeared comparable with that of the surface expression inhibition.

Inhibition of TNF-α-induced activation of NF-*k*B and degradation of IkBs by vitamin C

To test whether the inhibitory effect of vitamin C on the TNF-α-induced ICAM-1 expression was mediated *via* NF-

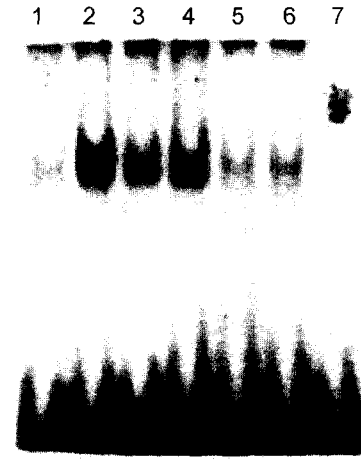

Fig. 3. The effect of vitamin C on the TNF-α-induced NF-*k*B activation in SK-N-SH cells. Cells (2×10^6 cells/mL) were pre-incubated at 37°C for 1 h, in the absence or presence of vitamin C (1, 2.5, 5 or 10 mM), and then treated with 5 ng/mL of TNF-α for 1 h. The nuclear extracts were then prepared and assayed for NF-*k*B by EMSA, on 6% polyacrylamide gel, using a DIG-labeled double-stranded oligonucleotide containing the NF-*k*B consensus sequence. The results illustrated are from a single experiment, and are representative of three separate experiments. Unstimulated (lane 1); TNF-α-stimulated (lane 2); TNF-α + Vitamin C (lanes 3-6). Binding competition assays were performed with a 100-fold excess of the unlabeled NF-*k*B oligonucleotide as a competitor (lane 7).

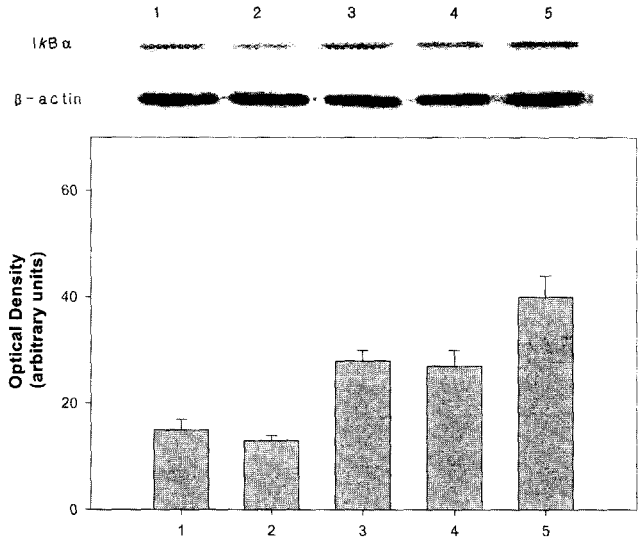

Fig. 4. Effect of vitamin C on the TNF-α-induced degradation of I*k*Bα in SK-N-SH cells. Cells (2×10^6 cells/mL) were pre-incubated at 37°C for 1 h, with or without vitamin C (1, 5 and 10 mM), and then treated with 5 ng/mL TNF-α for 1 h. The cytoplasmic extracts were prepared and assayed for I*k*Bα by western blot analysis. A typical result from three independent experiments is shown. Relative changes in the I*k*Bα protein bands were measured using densitometric analysis and optical densities (n=3), and are plotted in arbitrary units as the means S.E.M. Unstimulated (lane 1); TNF-α-stimulated (lane 2); TNF-α + Vitamin C (lanes 3-5).

*k*B, neuroblastoma cells were treated with various doses of vitamin C for 1 h before stimulation with TNF-α. The data showed that treatment with vitamin C resulted in a decrease of TNF-α-mediated NF-*k*B activation (Fig. 3). At 5 mM vitamin C, the TNF-α induced NF-*k*B activation was inhibited by 75%, as determined by densitometry scanning (data not shown). The specificity of the protein-DNA complex for the NF-*k*B sequence was demonstrated by competition with excess unlabeled NF-*k*B oligonucleotide.

To determine whether vitamin C affects the TNF-α-induced degradation of I*k*Bs, a western blot assay was performed. Analysis of cell extracts using I*k*B-specific antibodies showed that stimulation of the cells with TNF-α caused rapid degradation of I*k*Bα compared to untreated cells. When cells were treated with increasing concentrations of vitamin C (1-10 mM) before incubation with TNF-α for 1 h, the I*k*Bα protein levels were significantly increased, indicating that the degradation of I*k*Bα was inhibited after pretreatment of cells with vitamin C (Fig. 4). Vitamin C alone did not induce I*k*Bα degradation (data not shown).

DISCUSSION

Adhesion molecule expressions and interactions are involved in the initiation and propagation of inflammatory neurological diseases. Cytokines, such as TNF-α, IL-I and IFN-γ, may be released in the CNS by either infiltrating leukocytes or by resident cells, such as astrocytes and microglial cells (Owens *et al.*, 1994). Furthermore, the transfer of certain cytokine genes renders cells immunogenic, for example, neuroblastoma cells, by inducing phenotypic changes. These cytokines can induce, in adjacent cells, the expression of adhesion molecules, such as ICAM-1 and VCAM-1, all of which are mechanisms involved in leukocytes recruitment (Springer, 1994). Thus, factors affecting the expression of endothelial adhesion molecules, are important in regulating vascular inflammatory processes. The data presented in this study show that TNF-α treatment of neuroblastoma cells increases the expression of ICAM-1 and activation of NF-*k*B. Our data also demonstrate that vitamin C effectively inhibits TNF-α-stimulated mRNA and protein synthesis of ICAM-1.

Recently, the signaling pathway involved in TNF-α-induced ICAM-1 expression has been reported in many cell types. Previous studies have reported that TNF-α activated the phosphatidylcholine-specific phospholipase C (PC-PLC) to induce the activation of protein kinase C, the protein tyrosine kinase, and the NF-κB site of the promoter, followed by the initiation of ICAM-1 expression (Chen *et al.*, 2001; Johnson *et al.*, 1996). Agents that block NF-κB signaling and, hence, adhesion molecule expression and leukocyte-endothelial interactions *in vitro* (Weber *et al.*, 1995), also exert marked effects on inflammatory responses *in vivo* (Albelda *et al.*, 1994). Therefore, it is conceivable that vitamin C modulates the intracellular signaling involved in these signaling pathways. Since NF-*k*B has been shown to be essential for the gene expression of adhesion molecules, inhibition of this transcription factor was expected to inhibit the induction of NF-*k*B-dependent genes. In the present study, it was found that vitamin C inhibited the activation of NF-*k*B induced by TNF-α in human neuroblastoma cells. The concentration of vitamin C used in this study did not affect cell viability. Thus, the decreased level of NF-*k*B was not due to cell death. Based these findings our data suggest that vitamin C inhibited the expression of ICAM-1 induced by TNF-α via inhibition of NF-*k*B activation.

NF-*k*B activation is always preceded by proteolytic degradation of the inhibitory kB proteins (Henkel *et al.*, 1993; Finco and Baldwin, 1995). Several I*k*B proteins have been identified at the molecular level, and two major species, I*k*Bα and I*k*Bβ, were found to be degraded in response to external stimuli (Henkel *et al.*, 1993), but only I*k*Bα was degraded in response to TNF-α (Thompson *et al.*, 1995). Thus, the effect of vitamin C was investigated on the I*k*Bα degradation in TNF-α-stimulated neuroblastoma cells. This cytokine induced a rapid degradation of the I*k*Bα protein, followed by its recovery after stimulation.

Vitamin C inhibited IκBα degradation and nuclear translocation of NF-kB. These findings confirm the earlier studies using T cells and endothelial cells (Munoz *et al.*, 1997; Bowie and O'Neill, 2000). Our data, showing the vitamin C induced inhibition of the transcriptional activation of ICAM-1 could indicate that vitamin C inhibits the binding of NF-kB to the upstream regulatory promoter sequences of this gene. Since the effects of vitamin C on adhesion molecule expression were closely paralleled by IkB degradation, our data suggest that vitamin C inhibits TNF-α-induced neuronal cell activation by preventing DNA binding of NF-kB. However, our data totally ruled out the possibility that the inhibition of ICAM-1 expression by vitamin C was due to the affect of the NF-kB/IkB signaling pathway at the level (or upstream) of IkB kinase (IKK). Recent studies have shown that in response to TNF-α stimulation, IkBs are phosphorylated by IKK, ubiquitinated and proteolytically degraded, which allows NF-kB to translocate to the nucleus (Karin and Ben-Neriah, 2000).

Since our study has shown that vitamin C inhibits NF-kB/IkB signaling, vitamin C would also be expected to affect the NF-kB-dependent expression of many other inflammatory genes, such as IL-1 and IL-6 and TNF-α, in many cell types. Additionally, although the concentration of vitamin C used in this study was in the millimolar range, our observations are likely to have relevance during inflammation *in vivo*. It has been reported that cells transport both reduced and oxidized forms of vitamin C inside cells, but through two different mechanisms, a Na^+-dependent co-transporter for ascorbic acid, and a facilitative glucose transporter for dehydroascorbic acid (DHA) (Vera *et al.*, 1993; Tsukaguchi *et al.*, 1999; Rumsey *et al.*, 2000). Under physiological conditions, vitamin C circulates in the blood in its reduced form, ascorbic acid, at approximately 50 μM; however, cells accumulate a wide range of intracellular concentrations of vitamin C, up to 6 mM in mononuclear leukocytes. Although our data did not show the effect of the reduced form on cells, it is possible that the inhibition of TNF-α signaling was evident at intracellular low concentrations of vitamin C.

In the present study, vitamin C inhibited the TNF-α-induced expression of ICAM-1 and NF-*k*B activation. These data also demonstrate the potential utility of inhibiting the NF-*k*B signalling pathway in neurological, as well as other inflammatory diseases.

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