

# Sodium Ascorbate (Vitamin C) Induces Apoptosis in Melanoma Cells via the Down-Regulation of Transferrin Receptor Dependent Iron Uptake

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Sodium ascorbate (vitamin C) has a reputation for inconsistent effects upon malignant tumor cells, which vary from growth stimulation to apoptosis induction. Melanoma cells were found to be more susceptible to vitamin C toxicity than any other tumor cells. The present study has shown that sodium ascorbate decreases cellular iron uptake by melanoma cells in a dose- and time-dependent fashion, indicating that intracellular iron levels may be a critical factor in sodium ascorbate-induced apoptosis. Indeed, sodium ascorbate-induced apoptosis is enhanced by the iron chelator, desferrioxamine (DFO) while it is inhibited by the iron donor, ferric ammonium citrate (FAC). Moreover, the inhibitory effects of sodium ascorbate on intracellular iron levels are blocked by addition of transferrin, suggesting that transferrin receptor (TfR) dependent pathway of iron uptake may be regulated by sodium ascorbate. Cells exposed to sodium ascorbate demonstrated down-regulation of TfR expression and this precedes sodium ascorbate-induced apoptosis. Taken together, sodium ascorbate-mediated apoptosis appears to be initiated by a reduction of TfR expression, resulting in a down-regulation of iron uptake followed by an induction of apoptosis. This study demonstrates the specific mechanism of sodium ascorbate-induced apoptosis and these findings support future clinical trial of sodium ascorbate in the prevention of human melanoma relapse. *J. Cell. Physiol.* 204: 192–197, 2005. © 2005 Wiley-Liss, Inc.

Melanoma is an increasingly common and potentially lethal malignancy. It can escape immune surveillance through several mechanisms, including immune suppression by soluble inhibitory factors and the decreased expression of MHC molecules (Huber et al., 1992; Ferrone and Marincola, 1995; Mukherji and Chakraborty, 1995). Several efforts have been devoted to manage this malignancy without successful results. It has been reported that vitamin C is effective in preventing proliferation and inducing apoptosis of this malignant tumor (Bram et al., 1980). However, the specific mechanisms involved in induction of apoptosis remains unclear. We have recently reported that vitamin C induces apoptosis of melanoma via the caspase-8 independent manner (Kang et al., 2003). In addition to this, we also demonstrated that vitamin C can regulate the production of endogenous IL-18 from melanoma (Cho et al., 2003).

Meanwhile, transferrin receptor (TfR) is essential for cellular iron uptake via the serum transport protein, transferrin. Although the highest expression of TfRs is detected on the surface of erythroblasts, high levels of TfR expression has been also reported on the surface of some tumor cells including melanoma, implying that TfR may be involved in some functional regulation of tumor cells (Hedley et al., 1985; Sawyer and Krantz, 1986; Abe et al., 1992; Cavanaugh et al., 1999). The anti-TfR mAb has been found to inhibit the cellular uptake of iron and block tumor cell growth *in vitro* (Trowbridge and Lopez, 1982; Trowbridge, 1989). In addition, *in vivo* studies have demonstrated that anti-TfR mAbs have an anti-proliferative effect upon tumor cells (White et al.,

1990). Furthermore, Fassl et al. recently reported that transferrin ensures survival of ovarian carcinoma cell from the apoptosis that is induced by TNF family proteins, TNF- $\alpha$ , FasL, and TRAIL (Fassl et al., 2003). Altogether, these results suggest that TfR can play critical roles in the regulation of tumor cell growth, presumably through the regulation of iron uptake.

In fact, iron is a critical factor for the formation of various biological substances, such as hemoglobin, myoglobin, cytochromes, catalase, and peroxidase. In recent years, more insight has been gained about the direct correlation between intracellular iron levels and the regulation of immune responses including apoptosis (Kawabata et al., 1997; Wang et al., 1998).

In an attempt to find the possible mechanism of melanoma cell apoptosis induced by vitamin C, we investigated cell surface expression of TfR and intracellular

**Abbreviations:** TfR, transferrin receptor; DFO, desferrioxamine; FAC, ferric ammonium citrate.

Jae Seung Kang and Daeho Cho contributed equally to this work.

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iron levels, both of which are known to be critical factors in the induction of apoptosis.

We hypothesized that ascorbic acid might regulate TfR expression and iron uptake to induce apoptosis in malignant melanoma cells, and our data in this report demonstrate that ascorbic acid causes apoptosis of malignant melanoma cells *in vitro* by decreasing TfR surface expression and thus iron uptake. These changes of TfR expression seem to be regulated post-transcriptionally.

## MATERIALS AND METHODS

### Cells

B16 murine melanoma cells ( $1 \times 10^6$  cells/ml) were used as target cells. Cell lines were maintained in continuous log phase growth and cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS).

### MAb and chemicals

Monoclonal antibody to murine TfR (CD71) was purchased from Pharmingen (San Diego, CA). Sodium ascorbate, transferrin, desferrioxamine (DFO), and ferric ammonium citrate (FAC) were obtained from Sigma (St. Louis, MO).

### Measurement of TfR expression and apoptosis

FACS analysis was performed to detect TfR expression and apoptosis. Sodium ascorbate (10 mM) was added to B16 murine melanoma cells, and human peripheral blood monocytes ( $1 \times 10^6$  cells/ml) for 4 h. In the case of testing the effects of DFO, transferrin, and FAC, cells were pretreated with DFO (0.5 mM) or ferric ammonium citrate (FAC, 150 µg/ml) for 18 h and transferrin (0.01 mg/ml) for 2 h prior to the addition of sodium ascorbate. After treatments, cells ( $1 \times 10^6$  cells) were then collected and washed twice with PBS containing 0.05% BSA. After two washes, cells were incubated with FITC conjugated anti-mouse or human TfR mAb for 30 min on ice, and washed three times. A flow cytometer was used for analysis. For apoptosis assay, B16 murine melanoma cells, and human peripheral blood monocytes were treated with or without sodium ascorbate and washed twice with cold PBS and then resuspended in  $1 \times$  binding buffer at a concentration of  $1 \times 10^6$  cells/ml. Cells were then incubated with 5 µl of Annexin V-FITC at room temperature for 15 min in the dark. 400 µl of  $1 \times$  binding buffer was added prior to flow cytometric analysis. The Annexin V-FITC apoptosis detection kit was purchased from Pharmingen.

### RT-PCR

To evaluate whether the changes of the surface TfR expression following ascorbate treatment was due to the changes of RNA transcription, RT-PCR was done. Briefly, cells were harvested after treatment of 5 mM sodium ascorbate for 5, 30 min, 1, 2, 4, and 8 h, respectively and washed three times with PBS. RNA was extracted using Trizol (Gibco-BRL, Gaithersburg, MD) and cDNA was made using Superscript II RT (Gibco-BRL) and Oligo(dT) (Promega) as a primer. Then, TfR cDNA was made using 5'-TCA, GTT, TCC, GCC, ATC, TCA, GTC, A-3' (sense) and 5'-GCA, CCA, ACA, GC T, CCA, AAG, TCG-3' (antisense). β-Actin cDNA was amplified at the same time for quantitative comparison using 5'-CAG, GGT, GTG, ATG, G-3' (sense) and 5'-GCA, GGA, TGG, CGT, GAG, GGA, GAG-3' (antisense). PCR products were electrophoresed and the density of each band was read and compared.

### Measurement of intracellular iron levels

B16 murine melanoma cells ( $2 \times 10^7$  cells) were cultured for 4 h in the presence or absence of 10 mM sodium ascorbate. And then cells were washed three times with ice-cold PBS in order to stop the iron uptake process. After washing, the cells were treated with HNO<sub>3</sub> and H<sub>2</sub>O<sub>2</sub> in a 3:1 ratio for extraction of sample by the method of Thunell (1965) and the remainder was centrifuged to separate cytosolic and stromal fractions of the cells as previously described (Morgan, 1988). And then B16

melanoma-associated iron content was measured by inductively coupled plasma atomic emission spectrometry (ICP-AES).

### Intracellular staining of TfR (CD71)

The distribution of CD71 in B16 murine melanoma, during sodium ascorbate-induced cell death, was assessed by confocal microscope using the FITC conjugated rat anti-mouse CD71 Ab (Pharmingen, San Diego, CA). Cells ( $2.5 \times 10^6$ ) were cultured at 37°C, 5% CO<sub>2</sub> for 48 h. Media was removed and then 10 mM sodium ascorbate was added. After incubation with different time, 1, 2, 2.5, 3, and 4 h, cells were collected, washed with FACS buffer (0.05% BSA, and 0.02% sodium azide in PBS, pH 7.4), resuspended 15 ml of 1:1 mixture of 4% paraformaldehyde with FACS buffer, and then incubated on ice for 15 min. Cells were then washed, resuspended to 5 ml of permeabilization buffer (0.1% saponin, 0.05% sodium azide in PBS, pH 7.4) and incubated on ice for another 15 min. After incubation, 1 µg/µl of rat anti-mouse CD71-FITC Ab was added and cells were incubated with on ice for 30 min. Cells were then fixed with 4% paraformaldehyde and examined by confocal microscope at 525 nm.

### Statistical analysis

The statistical significance was determined by using ANOVA at  $P < 0.05$ . All results were given as a two-tail  $P$  value.

## RESULTS

### Sodium ascorbate induced-apoptosis on melanoma cells

We used the 10 mM sodium ascorbate in this experiment and it is relatively higher than physiological concentration in human beings. Therefore, we first examined whether 10 mM sodium ascorbate has the cytotoxic effects on human peripheral blood mononuclear cell (PBMC). As shown in Figure 1, we confirmed that 10 mM sodium ascorbate significantly induced apoptosis in B16 murine melanoma cells. However, it did not show the harmful effect on PBMC, nor induced apoptotic cell death.

### Reduction of intracellular iron levels

We have already reported the sodium ascorbate-induced apoptosis in B16 murine melanoma and we also confirmed it in this experiment (Fig. 1A). In addition, iron-induced apoptosis has been documented in other system (Kawabata et al., 1997; Wang et al., 1998). To investigate whether sodium ascorbate-induced apoptosis in B16 murine melanoma has the direct correlation with intracellular iron levels, the levels of B16 murine melanoma cells were measured after sodium ascorbate treatment. Surprisingly, sodium ascorbate reduced intracellular iron levels (Fig. 2A). To confirm the reduced intracellular iron levels involved in the induction of apoptosis, we tested the effects of DFO and FAC on sodium ascorbate-induced apoptosis. DFO chelates intracellular iron pools deleting available intracellular iron, and FAC enhances intracellular iron levels by increasing the iron uptake via nonspecific mechanism (Goto et al., 1983; Bottomley et al., 1985). Hence, these two chemicals are commonly used to study the roles of iron by modulating intracellular iron levels. As we expected, exposure to DFO significantly enhanced sodium ascorbate-induced apoptosis. In contrast, FAC significantly blocked the induction of apoptosis, indicating that intracellular iron levels are involved in sodium ascorbate-induced apoptosis (Fig. 2B).

### Down-regulation of TfR (CD71) expression

It has been suggested that two mechanisms of iron uptake exist in melanoma cells. One is a specific process of iron uptake by TfR-mediated endocytosis and the

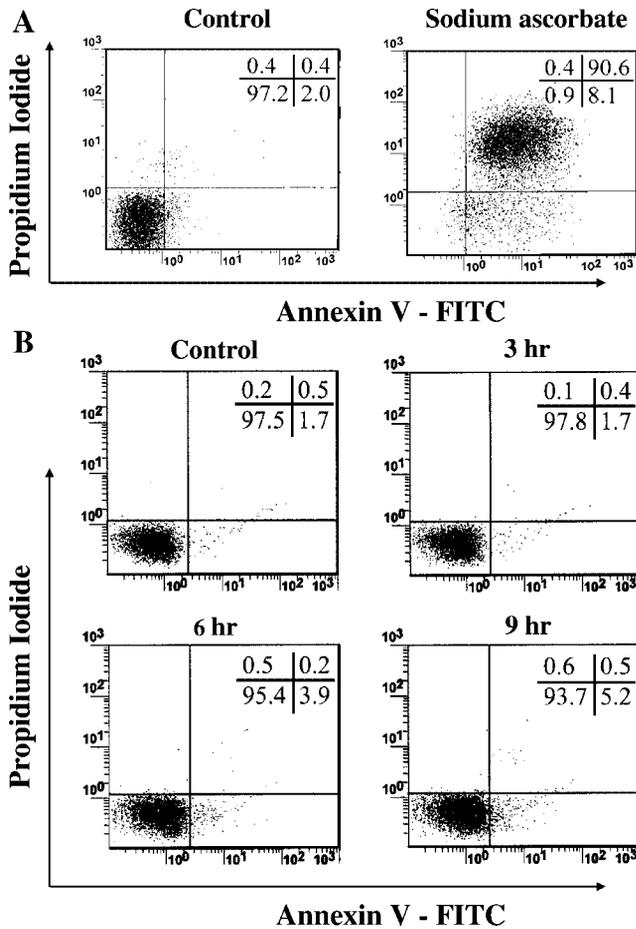


Fig. 1. Effect of sodium ascorbate on B16 murine melanoma and human peripheral blood mononuclear cells (PBMC). **Part A:** B16 murine melanoma cells ( $1 \times 10^6$ ) were cultured in the presence or absence of 10 mM vitamin C for 4 h. And then apoptotic cells were detected by staining with Annexin V-FITC. **Part B:** PBMC were isolated by Ficoll-Hypaque density gradient centrifugation of peripheral blood. PBMC were washed and then resuspended at  $1 \times 10^6$  cells/ml in RPMI 1640 with 10% FCS. After incubation at 37°C, 5% CO<sub>2</sub> for 12 h, 10 mM of sodium ascorbate was added and the cultured another 3, 6, and 9 h. Apoptotic cells were detected by staining with Annexin V-FITC.

other involves a nonspecific process of iron uptake at the plasma membrane (Bridges and Cudkovic, 1984; Richardson and Baker, 1992). To investigate pathways regulated by sodium ascorbate, cells were first treated with transferrin, which carries iron in plasma and interstitial fluids. The inhibitory effect of sodium ascorbate on reduction of intracellular iron levels was blocked by transferrin (Fig. 3A). Furthermore, treatment of B16 murine melanoma cells with transferrin prevented the induction of apoptosis, indicating that Tfr-mediated iron uptake should be inversely correlated with apoptotic induction (Fig. 3B).

Therefore, we tested whether sodium ascorbate down-regulates expression of Tfr. Following 2 h incubation with sodium ascorbate (10 mM), cultured B16 murine melanoma cells showed reduced Tfr expression in a dose and time-dependent (Fig. 4A,C), while ICAM-1 expression did not change (data not shown). It means that sodium ascorbate specifically reduces Tfr expression on the surface of melanoma cells. To clarify whether down-regulation of Tfr expression by sodium ascorbate is tumor specific, we investigated the changes of Tfr expression on human peripheral blood monocytes after treatment of the same concentration of sodium ascor-

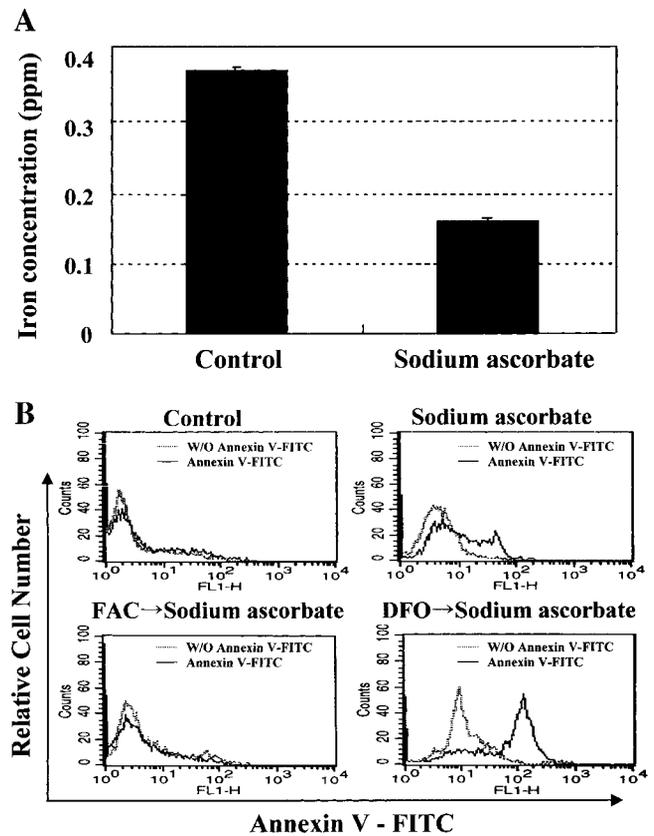


Fig. 2. Reduction of intracellular iron levels by sodium ascorbate. **Part A:** B16 murine melanoma cells ( $2 \times 10^7$  cells) were treated with 10 mM of sodium ascorbate for 4 h and used to detect intracellular iron levels. After washed three times with PBS, and then cells were treated with HNO<sub>3</sub> and H<sub>2</sub>O<sub>2</sub> in a 3:1 ratio. B16 melanoma-associated iron content was measured by inductively coupled plasma atomic emission spectrometry (ICP-AES). Values are the average  $\pm$  SD of triplicates. One representative experiment of four is shown. **Part B:** Cells ( $1 \times 10^6$  cells/ml) were exposed to media (control), DFO (0.5 mM), or FAC (150  $\mu$ g/ml) for 18 h. The cells were then washed and treated with sodium ascorbate (10 mM) for 4 h. Apoptotic cells were detected by staining with Annexin V-FITC.

bate. Interestingly, we have found that Tfr expression is increased in a time dependent manner (Fig. 4B). This indicates that reduction of Tfr expression by sodium ascorbate is specific process in tumor cells. Next, we examined the correlation of a down-regulation of Tfr expression and sodium ascorbate-induced apoptosis by performing of kinetic experiment. As a result, sodium ascorbate-induced apoptosis was preceded by a down-regulation of Tfr expression, indicating the correlation between apoptotic induction and Tfr reduction (Fig. 4D).

#### Post-transcriptional regulation of Tfr

It has been well documented that Tfr mRNA expression is tightly linked to intracellular iron levels. Under low iron concentrations, cells increase iron uptake through the enhancement of Tfr expression on the cell surface. This enhancement is mediated by increasing the stability of Tfr mRNA. In contrast, degradation of Tfr mRNA was detected in high levels of iron concentrations (Bridges and Cudkovic, 1984; Owen and Kuhn, 1987; Mullner et al., 1989). To analyze the mechanism underlying the sodium ascorbate induced reduction of Tfr expression, the level of mRNA for Tfr was

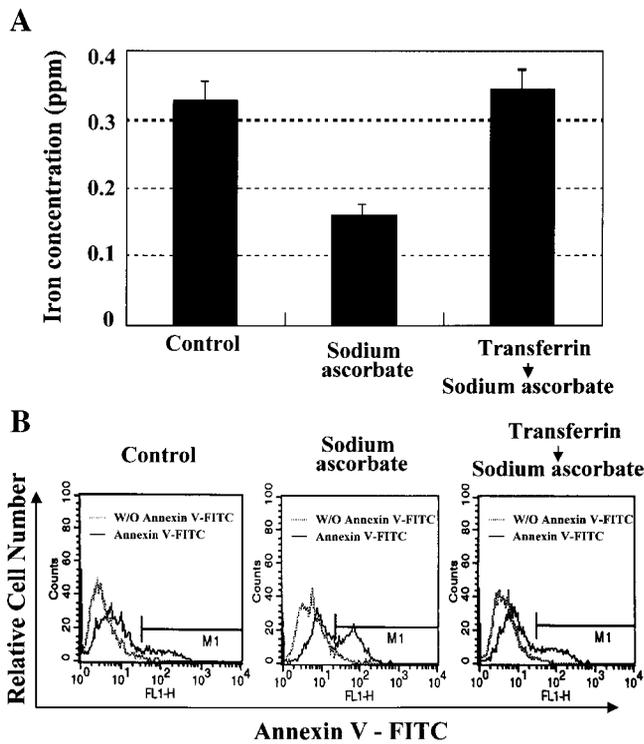


Fig. 3. The inhibitory effects of transferrin on sodium ascorbate-regulated intracellular iron levels and apoptosis. Cells ( $1 \times 10^6$  cells/ml) were incubated with transferrin (0.01 mg/ml) for 2 h followed by addition of sodium ascorbate (10 mM) for 4 h. After treatment, cells were washed and tested for intracellular iron levels and apoptotic cell death. **Part A:** intracellular iron levels. **Part B:** Apoptotic cell death. Values are the average  $\pm$  SD of triplicates. The data are representative of three similar experiments.

tested using a RT-PCR assay. We could extract mRNA from sodium ascorbate treated cells only for 30 min, 1 and 2 h, since there was no viable cells after 4 h under 10 mM of sodium ascorbate. And we have found that there was no significant change (data not shown). To examine TfR mRNA stability is maintained for longer, we decreased the concentration of sodium ascorbate from 10 to 5 mM. Surprisingly, no remarkable change in the level of TfR mRNA was detected in sodium ascorbate treated B16 murine melanoma cells, indicating that the reduction of TfR expression by sodium ascorbate is not mediated by regulating TfR mRNA stability (Fig. 5A). Next, we investigated the intracellular TfR distribution using a confocal microscope. Interestingly, intracellular staining of TfR showed that evenly distributed TfR in the cytoplasm of the control melanoma cells were localized in some site of the cytoplasm after exposure to sodium ascorbate, suggesting that vitamin C may interfere the transport of TfR to membrane surface (Fig. 5B). Therefore, the mechanism by which sodium ascorbate modulates TfR expression is complex and requires additional detailed investigation. Nevertheless, this system may be a good model to investigate regulation of TfR expression mediated other than degradation of mRNA.

## DISCUSSION

We have executed a study on the effect of sodium ascorbate (vitamin C) on the treatment and prevention of cancer, especially melanoma. As a result, we have reported that the production of endogenous IL-18, which is known as a critical factor for the immune escape

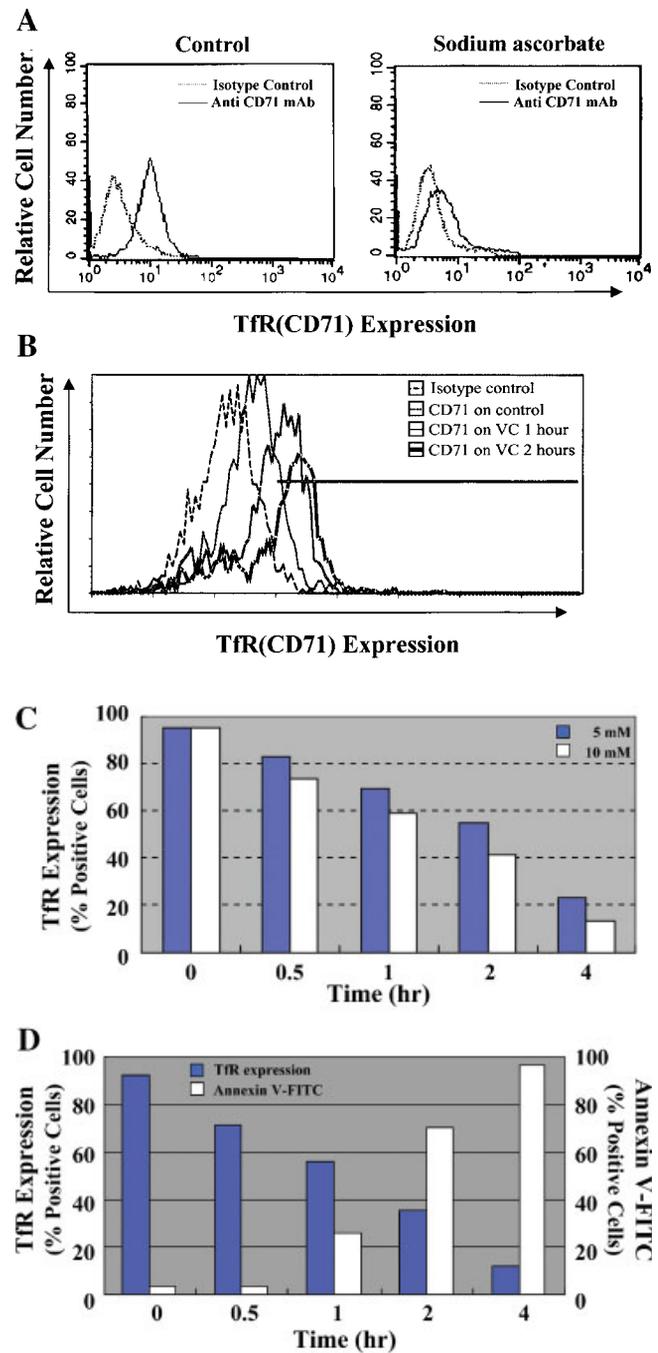


Fig. 4. Reduction of TfR expression by sodium ascorbate. **Part A:** After treatment of B16 murine melanoma cells with 10 mM of sodium ascorbate for 4 h, cells were collected and TfR expression was determined by FACS analysis. **Part B:** Human monocytes were purified from peripheral blood by Ficoll-Hypaque density gradient centrifuge. After cells ( $1 \times 10^6$  cells/ml) were cultured with sodium ascorbate for 1 and 2 h and then TfR expression was determined by FACS analysis. **Part C:** Cells ( $1 \times 10^6$  cells/ml) were cultured with sodium ascorbate for various time periods and concentrations as indicated. After culture, cells were harvested and TfR expression was determined by FACS analysis. **Part D:** Reduction of TfR expression (black bar) and induction of apoptosis by 10 mM of sodium ascorbate (white bar) were compared. The data are from one representative experiment out of three.

mechanism of melanoma, is down-regulated by sodium ascorbate (Cho et al., 2003). In addition, we also have documented that sodium ascorbate induces apoptosis on melanoma via the decrease of mitochondrial membrane potential and subsequent release of cytochrome C (Kang

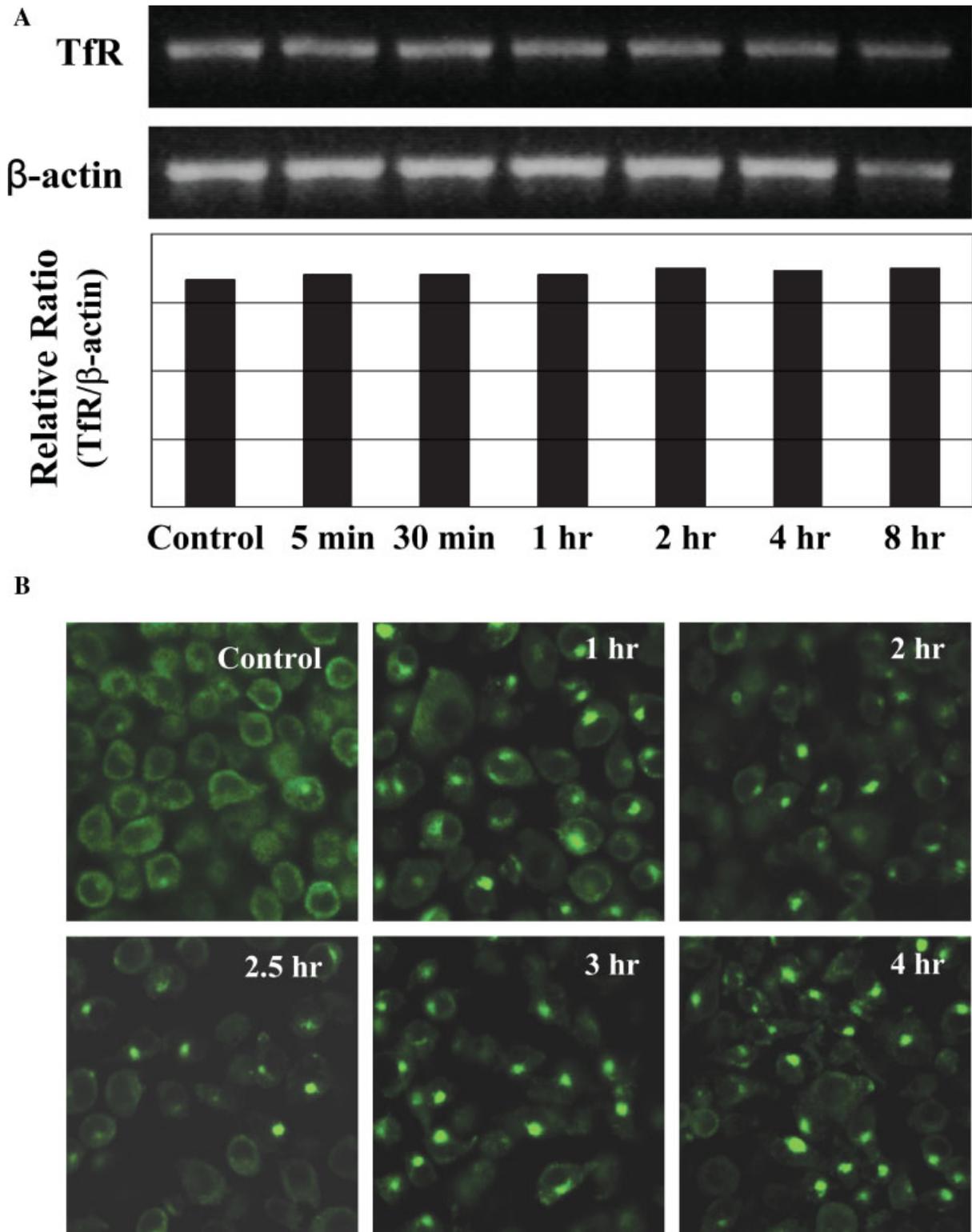


Fig. 5. Post-transcriptional regulation of TfR in melanoma cells by sodium ascorbate. **Part A:** Cells were harvested after sodium ascorbate (5 mM) treatment for 5, 30 min, 1, 2, 4, and 8 h, respectively, mRNA was extracted, cDNA was made, and TfR cDNA was amplified using appropriate primers. For normalization,  $\beta$ -actin cDNA was also amplified and compared. The density of TfR band did not change

remarkably compared to that of the control throughout the experimental period. A representative experiment from four is shown. **Part B:** Cells were incubated with 10% RPMI (control) or 10 mM of sodium ascorbate for 1, 2, 2.5, 3, and 4 h to detect the intracellular distribution of TfR (CD71). And then cells were stained with 1  $\mu$ g/ $\mu$ l of rat anti-mouse CD71-FITC and examined by confocal microscope at 525 nm.

et al., 2003). Moreover, there are several recent reports regarding the correlation of iron levels with apoptosis (Trowbridge and Lopez, 1982; White et al., 1990). Therefore, we did not exclude the possibility that there

might be another sodium ascorbate-induced apoptotic pathway and focused the relationship between the change of intracellular iron levels and sodium ascorbate-induced apoptosis in this study.

From the results of the present study, it now appears that sodium ascorbate decreases TfR-mediated iron uptake. However, it is not clear whether sodium ascorbate also regulates nonspecific processes of iron uptake at the plasma membrane. Usually, this nonspecific process of iron uptake occurs after the saturation of the transferrin-binding sites in TfR receptors or the down-regulation of TfR as occurs after FAC treatment. Incubation of cells with FAC results in the down-regulation of TfR but an accumulation of intracellular iron (Goto et al., 1983; Richardson and Baker, 1992). Since the concentration of iron in sodium ascorbate-treated melanoma cells remains at a low level, we hypothesize that sodium ascorbate may directly regulate nonspecific processes of iron uptake. Further studies will be needed to clarify this issue.

Is the down-regulation of TfR and iron uptake by sodium ascorbate a general pathway for inducing apoptosis? To answer this question, we tested other tumor cell lines such as IM-9 (multiple myeloma) and Raji (Burkitt's lymphoma), which are known to be sensitive to vitamin C toxicity. Similar results were documented in these tumor cell lines, suggesting that a down-regulation of TfR expression and iron uptake may be a common pathway to apoptosis by sodium ascorbate (data not shown). In contrast, other studies have reported sodium ascorbate enhanced intracellular iron concentration in U937 and THP-1 tumor cell lines and that this enhancement was associated with the induction of apoptosis (Laggner et al., 1999; May et al., 1999). Since iron metabolism is closely associated with reactive oxygen intermediates, we speculate that these paradoxical effects of vitamin C may be partially explained by taking into consideration of its bimodal action as an anti-oxidant, and an oxidant. Nevertheless, it is evident that regulation of TfR expression and iron uptake is a unique pathway of vitamin C induced apoptosis.

This study demonstrates the specific mechanism of sodium ascorbate-induced apoptosis and these findings support future clinical trial of sodium ascorbate in the prevention of human melanoma relapse.

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