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Original Research Communication

Enhanced Invasion of Tax-Expressing Fibroblasts into the Basement Membrane Is Repressed by Phosphorylated Ascorbate with Simultaneous Decreases in Intracellular Oxidative Stress and NF-κB Activation

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ABSTRACT

Invasion of rat fibroblastic cells Rat-1 through Matrigel was shown to be promoted by transfection with *tax* gene of human T-cell leukemia virus type 1. We found that an oxidation-resistant type of vitamin C (Asc), Asc-2-*O*-phosphate (Asc2P), inhibited the invasion of the *tax*-transfected Rat-1 cells (W4 cells). Intracellular Asc (Asc_{in}), after enzymatic dephosphorylation of administered Asc2P, was more abundant in W4 cells than in Rat-1 cells, and the ratio of dehydroascorbic acid versus Asc was increased in W4 cells but scarcely in Rat-1 cells, according to the enhanced level of intracellular reactive oxygen species (ROS_{in}) in W4 cells. Asc2P notably repressed the increases in both ROS_{in} and secretion of matrix metalloproteases (MMPs), but did not affect Tax protein expression in tax-transfectants. NF-κB activation, as evidenced by its translocation to the nucleus in W4 cells, was also repressed by Asc2P. Thus, the *tax*-promoted invasion together with the enhanced production of MMPs occurred with NF-κB activation and the increase in ROS_{in}, both of which were effectively reduced by Asc2P. These findings indicate the therapeutic efficacy of Asc_{in}-enriching agents for the prevention against tumor invasion in which ROS_{in} plays a major role. Antiox. Redox. Signal. 2, 727–738.

INTRODUCTION

HUMAN T-CELL LYMPHOTROPIC VIRUS TYPE 1 (HTLV-1) is the etiological agent of adult T-cell leukemia (ATL) (Poiesz *et al.*, 1980). Leukemic cells found in ATL patients often show severe invasion in the lymphoid organs and it is believed that the transcriptional activator coded in the X region of HTLV-1 (Tax) plays a central role in ATL leukemogenesis (Watanabe, 1997). Tax is reported to cause transformation of rodent fibroblasts (Pozzatti *et al.*, 1990; Tanaka *et al.*, 1990; Smith and Greene, 1991). Rat fibroblastic cells (Rat-1) transfected with a *tax* gene expression plasmid (W4 cells) undergo apoptosis by serum deprivation (Yamada *et al.*, 1994).

NF-κB activation would be also involved in cell transformation (Kitajima *et al.*, 1992; Matsumoto *et al.*, 1997; Coscoy *et al.*, 1998). The *tax* gene is associated with the acquisition of anchorage-independent formation of colonies in

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soft agar, and the ability to activate NF-κB is a prerequisite for transformation of rat fibroblasts (Yamaoka *et al.*, 1996). NF-κB is activated by diverse stimuli including tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), phorbol-12-myristate-13-acetate (PMA), UV light, and hydrogen peroxide, most of which are thought to act, at least in part, through the generation of reactive oxygen species (ROS) (Piette *et al.*, 1997; Li and Karin, 1999; Bowie and O'Neill, 2000).

In addition to ROS-mediated cell injury, a role of ROS in cellular signal transduction via redox regulation has received attention (Gius *et al.*, 1999). It is generally known that ROS is detrimental to tumor cells as shown in mechanisms shared by various anticancer agents or radiation therapy. Other studies indicate that tumor metastatic ability is enhanced by ROS and repressed by reduction of ROS (Toyokuni *et al.*, 1995). In addition, Tax stimulates nuclear translocation of NF-κB, which may involve altered cellular oxidation-reduction status (Los *et al.*, 1998). Recently, we found that some derivatives of vitamin C, the most rapid ROS scavenger, were useful as an antimetastatic agent and that intracellular vitamin C content was one of determinants for tumor invasiveness and metastatic ability (manuscript in press).

We used Rat-1 cells for the following reasons. Rat-1 cells are susceptible to transformation not only by Tax, but also by various nuclear and cytoplasmic oncoproteins with a low background of spontaneous foci (Yamaoka *et al.*, 1996). Moreover, Rat-1 cells can be grown in the presence of a high NF-κB activity as well as in the absence of it (Yamaoka *et al.*, 1998). In an attempt to clarify the role of Tax in the tumor invasion process, we investigated the relationship between the Tax-induced invasion phenotype and the oxidative stress.

MATERIALS AND METHODS

Cells

Rat-1 (rat fibroblast cells) and W4 (Rat-1 cells expressing Tax) cells, were grown in Dulbecco's modified Eagle minimum essential medium (D-MEM) supplemented with 10% dialyzed heat-inactivated fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY), 50 U/ml penicillin, and 50 μg/ml streptomycin. W4 cells were maintained in a medium containing 600 μg/ml G418 (Gibco BRL).

Invasion and haptotactic cell migration assay

Invasiveness into Matrigel (Albini *et al.*, 1987) (Collaborative Res. Bedford, MA) on the upper surface of microporous filters in a chemotactic chamber system or haptotactic migration toward fibronectin or laminin on the lower surface was determined as previously described (Albrecht-Buehler, 1977). Cells were incubated with or without L-ascorbic acid (Asc; Sigma St. Louis, MO) or Asc-2-*O*-phosphate magnesium salt (Asc2P; Showa Denko, Tokyo) at 0–500 μ*M* for 18 hr. Cells were then detached with trypsin to form a single-cell suspension in the complete medium (D-MEM supplemented with 10% FBS) to which Asc2P or Asc of indicated concentrations was also added. Aliquots (2×10^5 cells) were added to the upper compartments and incubated for 18–48 hr to allow cell motility in the presence of indicated concentrations of Asc2P or Asc. After incubation, noninvasive cells residing on the upper surface of the filter were thoroughly removed by wiping with several cotton swabs. Numbers of cells adhering to the lower surface were determined by microtiter photometry for mitochondrial dehydrogenase activity using the formazan dye-producing tetrazolium WST-1 (Ishiyama *et al.*, 1993) (Dojin, Kumamoto, Japan). Each assay was performed in triplicate, and independently repeated three times.

Protein extraction

Rat-1 or W4 cells were incubated with Asc2P in a manner similar to that described for the tumor invasion assay, washed carefully with cold PBS, and harvested with a rubber policeman in the presence of lysis buffer containing 50 m*M* Tris-HCl, pH 8.0, 4 m*M* ethylenediamine tetraacetic acid (EDTA), 150 m*M* NaCl, 1 m*M* phenymethanesulfonyl fluoride (PMSF), 1 m*M* dithiothreitol (DTT), 1% IGEPAL CA-630, and 1% sodium dodecyl sulfate (SDS). The cell suspension was kept at −20°C for 3 hr and then the cells were lysed with a rocking rotor for 1

hr at 4°C. The suspension was centrifuged at 15,000 rpm at 4°C for 5 hr. The resulting precipitate was soaked in 80% acetone, and the acetone extract was air-dried producing an extracted sample.

Western blot analysis of Tax

Protein extracts were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto nitrocellulose membranes using a semidry blotter (Atto, Tokyo). The filter was then applied sequentially with mouse monoclonal anti-Tax antibody, MI73 (Mori *et al.*, 1987), and secondary antibodies conjugated with horseradish peroxidase and detected with an enhanced chemiluminescence (ECL) detection system (Amersham, Uppsala, Sweden). The density of the bands was measured with a laser densitometer.

Electrophoretic mobility-shift assay

Rat-1 or W4 cells were treated with or without Asc2P of 300 μM in a manner similar to that described for the tumor invasion assay. Nuclear protein extraction was performed as described previously (Yang *et al.*, 1995). Electrophoretic mobility-shift assay (EMSA) was carried out with two sequence-specific gel-shift assay system of NF-κB (Promega, Madison, WI). The double-stranded oligonucleotide probe containing the specific wild-type DNA binding domain for NF-κB was 5′-TTTC-TAGGGACTTTCCGCCTGGGGACTTT-CCAG-3′, and the two underlined sites were changed from GGG to CTC to generate a mutant-type oligonucleotic probe (Takara, Tokyo). The oligonucleotides were labeled with [α-^{32}P]dATP (Pharmacia) using the Klenow fragment of DNA polymerase I (Takara) and purified using a gel-filtration column (MicroSpin G-25, Pharmacia).

Immunocytochemical staining of NF-κB

Cells were cultured on coverslips at a density of 1.26×10^3 cells/mm^2 in an eight-chamber slide system (Nunc, Roskilde, Denmark). The cells were treated under the same conditions as described for the tumor invasion assay, washed with phosphate-buffered saline PBS (−), and fixed with 4.5% formaldehyde for 15 min. Cytolysis was then conducted with 0.5% Triton X-100 at room temperature for 20 min. The specimen was incubated at 37°C for 1 hr with primary antibodies against NF-κB p65 (RelA) (Santa Cruz Biotechnology) diluted at a rate of 1:100 with PBS(−) containing 1% bovine serum albumin (BSA). The samples were then washed three times with 0.05% Triton X-100 and incubated at 37°C for 40 min with secondary antibodies, fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG. The preparations were washed three times with PBS(−) and mounted onto slides using PermaFluor aqueous mounting medium (Immunon, Pittsburgh, PA). The slides were examined on a laser scanning confocal fluorescence microscope (MRC-600; Bio-Rad, Richmond, CA) equipped with argon laser as a light source.

Assay of enzymatic degradation of basement membranes

Supernatant of conditioned medium derived from cells cultured in the absence or presence of Asc2P of indicated concentrations was analyzed by zymography and Western blotting. Conditioned media were concentrated by ultrafiltration (Ultrafree-MC, Millipore), and analyzed by gelatin zymography as previously described (Ballin *et al.*, 1988). Briefly, enzyme-containing samples were electrophoresed under nonreduced conditions using SDS-polyacrylamide gel containing 0.2% gelatin (8% total acrylamide). The gels were washed with 2.5% Triton X-100 for 1 hr, incubated in 0.05 *M* Tris-HCl buffer, pH 7.5, containing 0.15 *M* NaCl, 0.01 *M* $CaCl_2$, and 0.02% NaN_3 for 12 hr at 37°C, and stained with Coomassie Brilliant Blue R250. Gelatinolytic bands were size-calibrated with molecular weight standard mixture of proteins (Bio-Rad) and were identified for gelatinase-A (MMP-2) and gelatinase-B (MMP-9). Western blot analysis for MMP-2 or -9 protein was carried out under the same procedures as described for Tax except that anti-MMP-2 and anti-MMP-9 antibodies (Santa Cruz Biotechnology) were used. Band intensities were measured by use of image analysis software (NIH image).

In situ *detection of cellular oxidative stress*

Treatment of cells with Asc2P was carried out as described for the invasion assay. Cells were grown in the presence or absence of Asc or Asc2P, rinsed twice with PBS(−), and then placed in phenol red-free MEM +10% FBS containing 10 μM 6-carboxy-2′,7′-dichlorodihydrofluorescein (CDCFH) (Molecular Probes, Eugene, OR) (Haugland, 1996). After 90 min of incubation, the supernatant was transferred to a new microplate to quantify the extracellular oxidative stress. Following rinsing, phenol red-free fresh medium was added to the plate well for measurement of the intracellular oxidative stress. The fluorescence intensity was measured with a fluorescence plate reader (CytoFluor 2350; Millipore) with excitation and emission wavelengths of 480 nm and 530 nm, respectively.

Electron spin resonance spectroscopy

Cells were incubated with Asc2P as described for the invasion assay, washed carefully with PBS(−) three times, and harvested with a rubber policeman. Cell suspensions were then lysed with Triton X-100 in PBS(−) and homogenized with a Potter-type Teflon homogenizer using 10 strokes on the ice. The cell extract was mixed with a solution of the spin-trapping reagent, DMPO (Labotec, Tokyo), vortexed for 10 sec, and immediately transferred into a quart flat cell. Electron spin resonance (ESR) spectrum recording was started exactly 60 sec after DMPO addition using an ESR spectrometer (X-band, JEOL JES-FR30) at a constant room temperature (4 mW microwave power, 335.5 ± 5 mT modulation amplitude, and 1 sec time constant). Reference samples of $MgO:Mn^{2+}$ were used for determination of g values and relative signal intensities. Ascorbyl radical (Asc·) signal shows an asymmetric doublet signal with a line width of 0.18 mT centered at $g = 2.007$, being in accord to the values previously reported (Morishige *et al.*, 1983).

Intracellular Asc concentrations

Cells were treated with Asc2P or Asc as described for the invasion assay, gently rinsed to remove extracellular substances, and thoroughly crushed with a Potter-type Teflon homogenizer on the ice. The high-performance liquid chromatography (HPLC) technique was used as previously described (Furumoto *et al.*, 1998). Asc, Asc2P, and DehAsc were separated as shown by retention times of 3.4, 6.5, and 20.0 min, respectively, under the chromatographic conditions employed.

Statistical analysis

Experimental values are expressed as the mean ± SD. Student's t-test was used to evaluate the significance of differences between test groups, and the criterion of statistical significance was taken as $p < 0.05$.

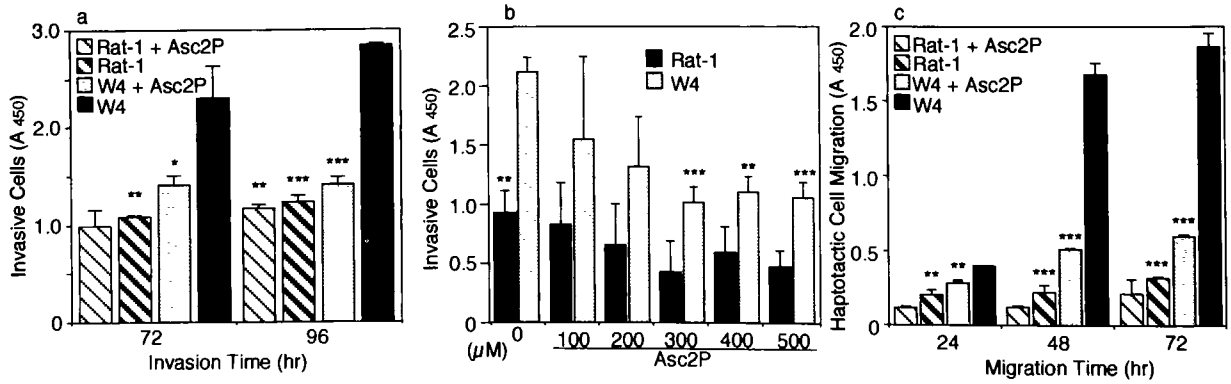

FIG. 1. Effects of L-ascorbic acid-2-*O*-phosphate (Asc2P) on tumor cell invasion (a and b) or migration (c) of rat fibroblastic cells Rat-1 and their tax-transfectants W4. Invasive or migrative cells were counted using WST-1 dye. (**a**) Invasion time-related effects of Asc2P. ***$p < 0.001$; **$p < 0.02$; *$p < 0.05$ as compared with the nontreated W4 cells. (**b**) Effects of Asc or Asc2P on invasion. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$, as compared with the nontreated W4 cells. (**c**) Effects of Asc2P on migration. ***$p < 0.001$; **$p < 0.01$, as compared with the nontreated W4 cells. The data are typical of independent experiments with chambers in triplicate, the S.D. of which is represented by the vertical bar.

RESULTS

Effects of Tax on tumor invasion

To examine the effects of Tax-induced transformation of cells on the invasion stage intrinsically commanding the metastasis, the invasive ability of rat fibroblastic cells and their *tax*-expressing transfectants through the reconstituted basement membrane Matrigel was assayed. Because Rat-1 parent cells, which are derived from the normal connective tissue, exhibit low invasive ability, the incubation period for the invasion was lengthened (24–72 hr) over that usually employed (2–4 hr). The number of invasive cells increased in a time-dependent manner for both untransfected (Rat-1) and *tax*-transfected (W4) cells (Fig. 1a). Invasion of W4 cells was enhanced about 2- to 8-fold relative to that of Rat-1 cells. Western blot analysis using Tax-specific antibodies confirmed expression of Tax protein in W4, but not in Rat-1 cells (Fig. 2). Asc2P treatment was shown to inhibit invasion of Rat-1 cells to 80% at 48 hr of invasion time, and invasion of W4 cells to 50% at 96 hr, relative to cells without Asc2P treatment (Fig. 1a). Asc2P at 300 μM, which can effectively inhibit invasion, did not affect cell viability or growth for either cell type, and 5 mM Asc2P failed to affect cell viability or growth (data not shown). Thus, the invasion-inhibitory effects of Asc2P are not attributable to direct cytotoxic action. The native type of vitamin C, ascorbic acid (Asc), also exhibited anti-invasive activity in W4 cells, although to a lesser extent than Asc2P did. However, Asc had no effect in untransfected Rat-1 cells (data not shown). Levels of Tax expression in *tax*-transfectants was not affected by addition of Asc2P, as shown by the Western blot analysis, suggesting that Asc2P suppresses invasion through targeting a cellular component that is also targeted by Tax (Fig. 2).

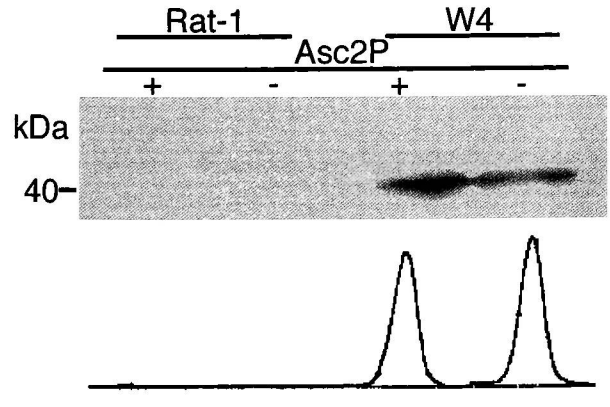

FIG. 2. Effects of treatment with Asc2P of 300 μM for 18 hr on Tax expression in Rat-1 and W4 cells as assessed by Western blot using anti-Tax Ab and HRP-conjugated anti-IgG Ab. The experimental conditions were similar to those described for the invasion assay in Fig. 1. (**Top**) Electrophorogram of the Tax protein probed with anti-Tax antibodies. (**Bottom**) Densitogram of Tax protein band levels.

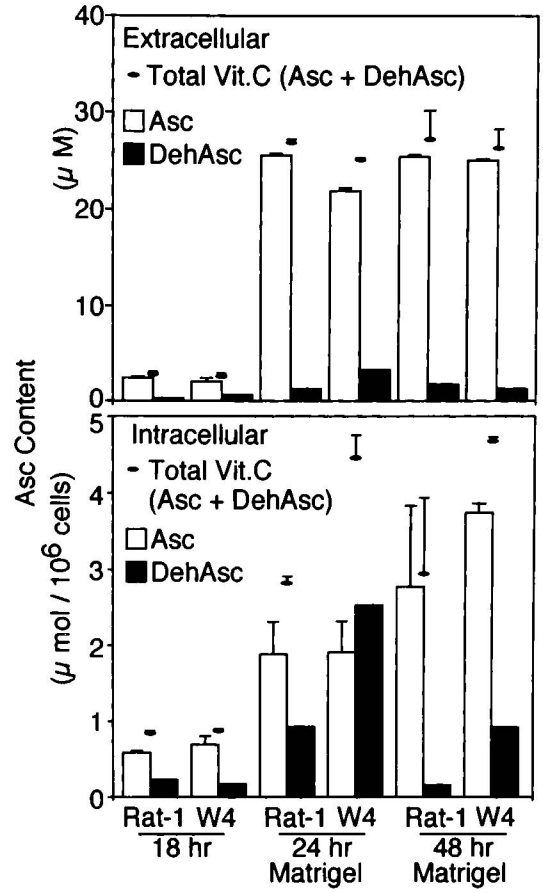

FIG. 3. Intracellular and extracellular contents of Asc or DehAsc as quantified by coulometric ECD/HPLC. Rat-1 and W4 cells were exposed to Asc2P for 18 hr as described for the invasion assay on a Matrigel precoated dish. Asc or DehAsc was not detectable for cells that were not exposed to Asc2P. Extracts from cells or the extracellular media from each group were quantified for Asc or DehAsc contents, the S.D. of which is represented by the vertical bar.

Effects of Asc2P on haptotactic cell migration

Cell migration toward the ECM component fibronectin was also suppressed by pretreatment with Asc2P, and this inhibition was

greater in W4 cells than in the parental Rat-1 cells (Fig. 1c).

Intracellular Asc concentrations

Rat-1 or W4 cells were treated with Asc2P in a manner similar to that done for the invasion assay. Subsequent analysis with HPLC and coulometric ECD detection showed that intracellular accumulation of Asc resulting from Asc2P administration was more marked in W4 cells than in Rat-1 cells (Fig. 3). Intracellular concentration of total vitamin C, Asc + DehAsc, were time-dependently elevated in both cell types, and the ratio of DehAsc versus Asc was increased in W4 cells compared to Rat-1 cells (Fig. 3). There was no significant difference between the two cell types in DehAsc reductase activity regardless of Asc2P addition (data not shown), suggesting that the abundance of intracellular DehAsc in W4 cells may be attributed to a Tax-linked increase in intracellular oxidative stress but not to decreased regeneration of vitamin C.

Promotive effects of Tax on NF-κB activation in W4 cells

The effects of Tax and/or Asc2P on NF-κB expression were assessed by a gel-shift assay (Fig. 4). DNA–NF-κB complexes were detected in the nuclei of *tax*-transfected W4 cells but scarcely in those of Rat-1 cells with a wild-type but not with a mutant κB DNA probe. NF-κB-specific DNA binding was decreased by Asc2P treatment in W4 cells. Immunocytochemical staining further confirmed reduced nuclear translocation of RelA in W4 cells treated with Asc2P (Fig. 5).

Effects of Tax and/or Asc2P on degradation of the extracellular matrix

The effects of Tax and/or Asc2P on extracellular matrix (ECM) degradation catalyzed by matrix metalloproteases (MMPs) necessary for the invasive process were examined by gelatin zymography. Gelatinolytic activity was exhibited by secretions from Rat-1 and W4 cells; the major activity was associated with bands of 72 kDa (gelatinase A or MMP-2) and 92 kDa (gelatinase B or MMP-9) in the

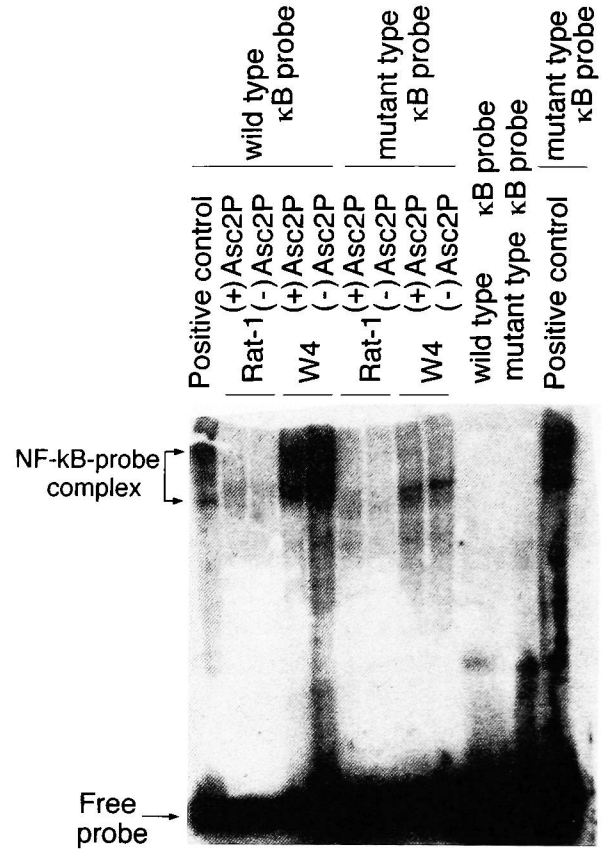

FIG. 4. Effects of Asc2P on NF-κB binding to DNA probes shown with Rat-1 and W4 cells. Nuclear extract from HeLa cells was used as a positive control. Specificity of the retarded bands was tested by use of a wild-type or mutant κB probe. Rat-1 and W4 cells were treated with 300 μ*M* Asc2P for 18 hr as described for invasion assay. Nuclear extracts were prepared and analyzed by EMSA using ^{32}P-labeled κB probes.

electrophorogram. Both activities of gelatinase were markedly decreased in secretions from Asc2P-treated cells, and the subsequent densitometry showed that Asc2P inhibits the production of MMP-9 to 80.0% in W4 cells (Fig. 6a). Production of MMP-2 and -9 was also determined with Western blotting using polyclonal antibodies directed against pro-MMP-2 and pro-MMP-9, demonstrating Asc2P-mediated inhibition of MMP-2 and -9 production to 44.0% and 41.0%, respectively (Fig. 6b,c).

Fluorographic analysis of oxidative stress

To examine whether Asc or Asc2P could influence ROS levels in Rat-1 and W4 cells, we quantified degrees of cellular oxidative stress by fluorometry using CDCFH, a dichlorodihy-

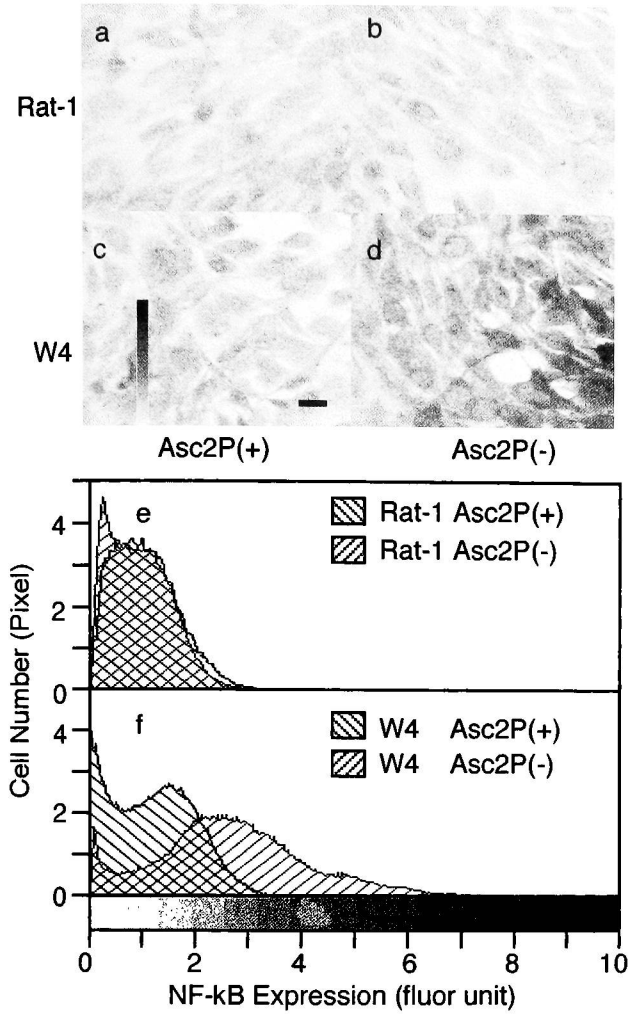

FIG. 5. Effects of Asc2P on NF-κB activation in Rat-1 and W4 cells as shown by immunocytochemical staining (anti-NF-κB p65-Ab/anti-IgG-Ab/FITC). The microscopic fields shown are typical of 12 independent fields of stained cells undergoing each treatment. Rat-1 (**a** and **b**) and Wr (**c** and **d**) cells. (**a** and **c**) Cells treated with Asc2P of 300 μM for 18 hr. (**b** and **d**) Control. (**e** and **f**) Graphic display shows semiquantitative histogram analysis where dispersion of colors forms darkness to brightness on the abscissa and the number of pixels found at each lightness value on the ordinate. The scale indicates 50 μm.

drofluorescein derivative, as a redox indicator (Haugland, 1996). The level of intra- and extracellular ROS in W4 cells was shown to be higher than in the Rat-1 cells following a 90-min incubation as shown by fluorometry (Fig. 7). In particular, the intracellular ROS level in W4 cells was significantly ($p < 0.05$) higher than in Rat-1 cells. Addition of 300 μM Asc2P, a dose with invasion-inhibitory properties (Fig. 1), markedly decreased the intracellular ROS level in W4 cells.

ROS-scavenging activity of Asc or Asc2P

We quantified hydroxyl radicals (HO·) and ascorbyl radicals (Asc·) in Rat-1 or W4 cells by ESR spectroscopy. ESR spectra typical of DMPO spin adducts with HO· were observed in both Rat-1 and W4 single-cell suspensions (Fig. 8). This signal was diminished by treatment with the HO· scavenger dimethyl sulfoxide (DMSO) and by pretreatment of W4 cells with Asc2P, suggesting that the enrichment

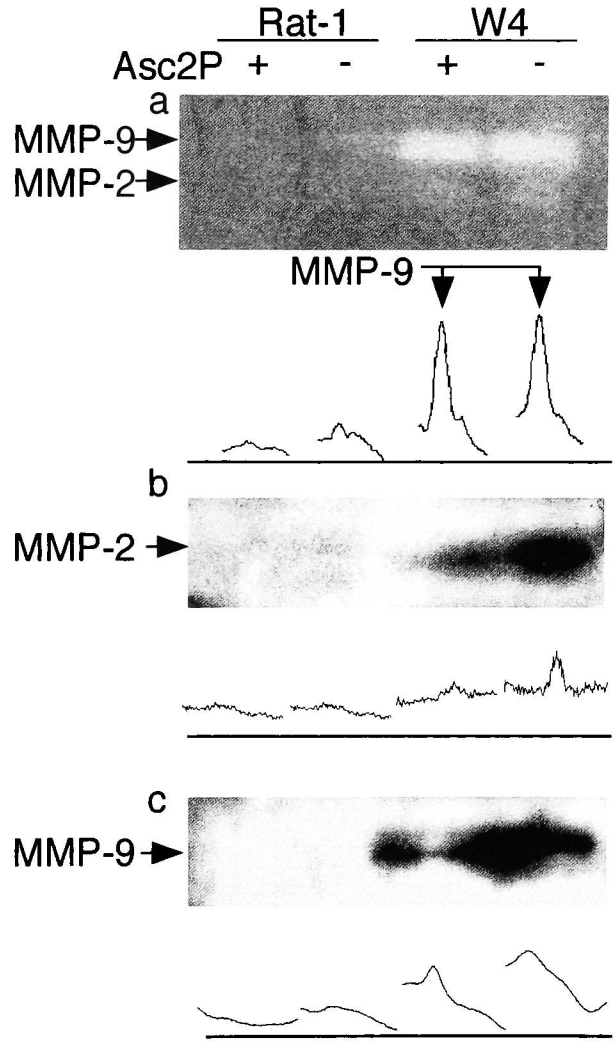

FIG. 6. Zymograms by PAGE using gelatin as the substrate of conditioned culture media derived from Rat-1 and W4 cells with or without Asc2P (a). Enzyme-digested regions were identified as white bands against the dark background. Effects of Asc2P of 300 μM on gene expression of MMP-2 (**b**) and MMP-9 (**c**) in Rat-1 and W4 cells were assessed by Western blot using anti-MMP-2 or -9 primary antibodies and HRP-conjugated anti-IgG secondary antibodies. Band intensities were measured by use of image analysis software (NIH image).

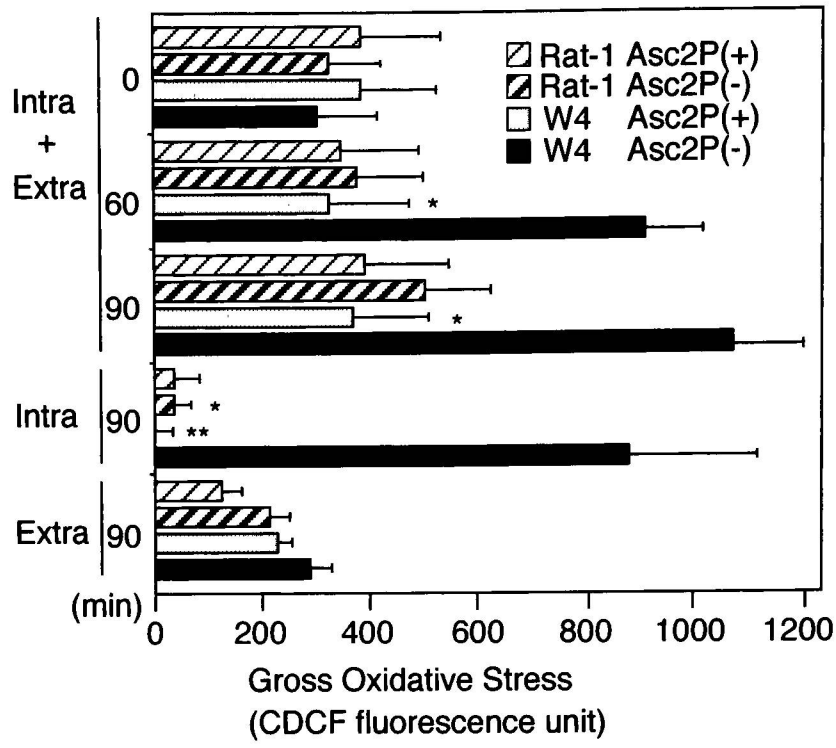

FIG. 7. Effects of Asc2P on the intra- or extracellular oxidative conditions of Rat-1 and W4 cells. Cells were pretreated with or without Asc2P, transferred into a 96-well plate, and given CDCFH-DA, and incubated for 60 and 90 min, followed by measurement of absorbance at 485 nm. $^{**}p < 0.05$; $^{*}p < 0.10$, as compared with the nontreated W4 cells. Fluorometric data shown are typical of three independent experiments, each conducted in triplicate, the S.D. of which is represented by the vertical bar.

of intracellular Asc following Asc2P addition resulted in increased scavenging of intracellular HO· Asc2P diminished DMPO/HO· spin adducts in a concentration-dependent manner. Asc2P pretreatment also induced the emergence of an asymmetric doublet signal, as previously reported for Asc· (Morishige, 1983).

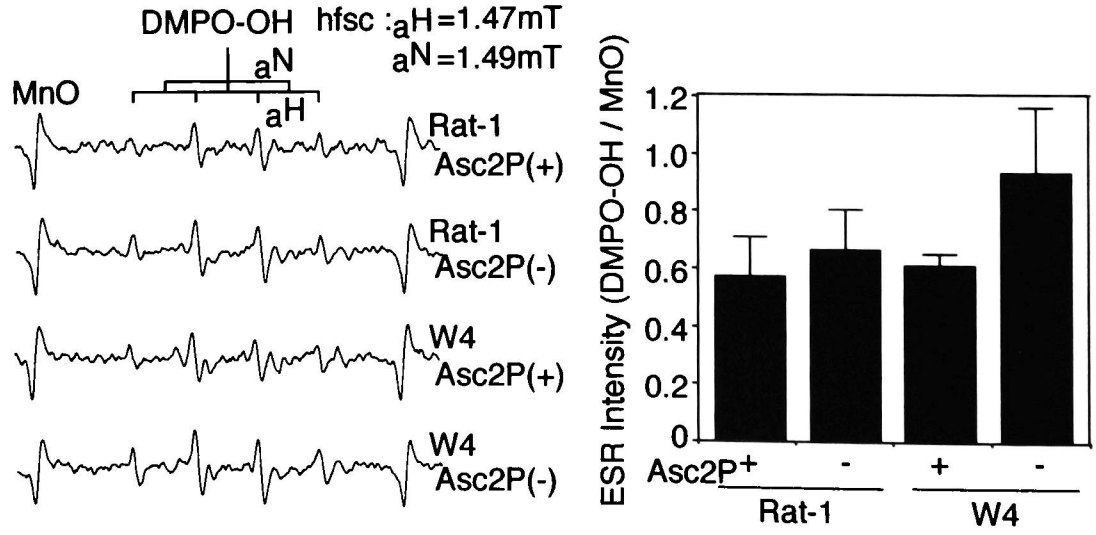

FIG. 8. Effects of hydroxyl radical generation in Rat-1 and W4 cells. ESR spectrum recording was started exactly 60 sec after addition of the spin-trapping agent DMPO to the cell homogenate. Cells were pretreated for 18 hr with Asc2P and rinsed prior to measurement. Relative intensities of ESR signals of DMPO-OH spin adducts versus those of MgO:Mn^{2+} are expressed.

DISCUSSION

The present study revealed that: (1) Tax expression significantly enhanced the invasive phenotype of Rat-1 cells (Fig. 1a,b); (2) Tax expression (Fig. 2) promoted the activation of NF-κB (Figs. 4 and 5), chemotactic properties (Fig. 1c), constitutive generation of ROS (Figs. 7 and 8), and gelatinolytic activity of type-IV collagenases (particularly MMP-9) (Fig. 6); and (3) pretreatment with Asc2P suppressed most of the above-mentioned properties of Tax-expressing cells.

Our results indicated that Asc2P by itself did not affect Tax expression, and that Asc2P inhibits DNA binding of NF-κB, suggesting that Asc2P exerted its effect at a step downstream of *tax* gene expression and likely upstream of the DNA binding of NF-κB. It is important to note that Tax was previously shown to induce an oxidative state in cells (Schreck *et al.*, 1992). Recent reports have demonstrated that expression of Tax in T lymphocyte cell lines resulted in increased cellular adherence/invasion of Matrigel filters (Trihn *et al.*, 1997). MMP-2 and/or MMP-9 are believed to play a significant role in the invasion of the basement membrane by tumor cells (Stearns *et al.*, 1997; Sugiura *et al.*, 1998). NF-κB binding sites are present in the promoter of the MMP-9 gene, but not that of the MMP-2 gene, which could explain an independent expression pattern of both proteinases (Huhtala *et al.*, 1991). Oxidant stress led to an increase in MMP activation (Rajagopalan *et al.*, 1996). Expression of MMP-9 was inhibited by treatment with antioxidants, *N*-acetyl-cysteine (Galis *et al.*, 1998), or curcumin (Lin *et al.*, 1998). Coexistence with macrophages (Mukai *et al.*, 1987) or PMA-pretreatment (Nonaka *et al.*, 1993) accelerated tumor invasion and metastasis. These observations indicate that ROS in tumor cells may play a critical role in the development of metastasis. NF-κB can be activated by Tax (Hiscott *et al.*, 1997). NF-κB activation has also been reported to be associated with metastatic phenotype (La Porta and Comolli, 1998; Dong *et al.*, 1999; Meyskens *et al.*, 1999; Wang *et al.*, 1999), regulation of extracellular matrix-degrading activity (He, 1996; Kheradmand *et al.*, 1998; Takeshita *et al.*, 1999; Xu *et al.*, 1999), and luminal environment by nutrient-derived oxidants (Aw, 1999; Sen, 1999), all of which function as invasion/metastasis promoting factors. The antioxidant pyrrolidine dithiocarbamate (PDTC) can block the mobilization of NF-κB into the nucleus by Tax (Schreck *et al.*, 1992). DNA-binding activity of NF-κB is inhibited by certain antioxidants such as quercetin (Musonda and Chipman, 1998), PDTC, *N*-acetyl-cysteine (Meyer *et al.*, 1993), vitamin E (Suzuki and Packer, 1993), and EPC-K1, a phosphodiester compound of vitamin C and vitamin E (Hirano *et al.*, 1998). Furthermore, invasive and metastatic properties of malignant cells are reportedly inhibited by oxygen radical scavengers, such as r-hSOD (Nonaka *et al.*, 1993), DMSO, and allopurinol (Salim, 1992), and by redox-regulating agents such as glutathione (Anasagasti *et al.*, 1998) and *N*-acetylcysteine (Albini *et al.*, 1995). Transfection of sarcoma cells with an antisense cDNA of Cu-Zn SOD promotes invasion and metastasis (Tanaka *et al.*, 1997). Thus, the level of ROS may be related to tumor invasion and metastasis as well as tumor promotion and carcinogenesis. The initial oxidative product of Asc, ascorbyl radical (Asc·), enhanced the DNA-binding activity of NF-κB in Jurkat T-cells stimulated by TNF-α (Munoz *et al.*, 1997). The facts that Tax activates transcription via several *cis*-regulatory elements within the cellular promoters, including the cyclic AMP response element (CRE) and the NF-κB binding sites (Mesnard and Devaux, 1999), and that NF-κB is involved in the maintenance of Tax-induced malignant phenotype (Kitajima *et al.*, 1992; Yamaoka *et al.*, 1996), together with the role of ROS in Tax-mediated NF-κB activation strongly implicate the link.

Finally, the cellular uptake of vitamin C, especially DehAsc, is promoted by Tax (Fig. 3), which may help cells to avoid the increased risk of oxidative stress. Treatment with vitamin C could be considered as one of the therapies for cancer patients (Head, 1998). Recent studies show that the transport of dehydroascorbic acid by glucose transporters is one of the means by which tumors acquire vitamin C, and indicate the oxidation of Asc by superoxide anion produced by cells in the tumor stroma as a mechanism for generating the transportable form of the vitamin C (Agus *et al.*, 1999). Increased trans-membrane uptake of DehAsc,

preferentially over Asc, has been shown to occur in some tumor cell types (Washko *et al.*, 1993). DehAsc reductase activity levels in *tax*-transfected and parental cells were unaltered as analyzed by spectrophotometric assay (data not shown).

Thus, our findings suggest that promotion of ROS generation by Tax and subsequent NF-κB activation could be responsible for enhanced invasive phenotype of *tax*-expressing cells. This process is suppressed by Asc2P through the enrichment of intracellular vitamin C. These findings also illustrate the importance of ROS in tumor invasion.

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ABBREVIATIONS

Asc, L-Ascorbic acid; Asc2P, ascorbic acid-2-*O*-phosphate; Asc_{in}, intracellular ascorbic acid; CDCFH-DA, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxy-methyl ester); DehAsc, dehydroascorbic acid; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; ECD, electrochemical detection; ECL, enhanced chemiluminescence; ECM, extracellular matrix; EMSA, electrophoretic mobility-shift assay; ESR, electron spin resonance; HRP, horseradish peroxidase; MMP, matrix metalloprotease; NF-κB, nuclear factor kappa B; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; WST-1 dye, (4-[3-(4-iodo-phenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate.

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