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Proteomic Analysis of Tumor Tissue after Treatment with Ascorbic Acid

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***Abstract*—**Tumor cells have an invasive and metastatic phenotype that is the main cause of death for cancer patients. Tumor establishment and penetration consists of a series of complex processes involving multiple changes in gene expression. In this study, intraperitoneal administration of a high concentration of ascorbic acid inhibited tumor establishment and decreased tumor mass in BALB/C mice implanted with S-180 sarcoma cancer cells. To identify proteins involved in the ascorbic acid-mediated inhibition of tumor progression, changes in the tumor proteome associated with ascorbic acid treatment of BALB/C mice implanted with S-180 were investigated using two-dimensional gel electrophoresis and mass spectrometry. Twenty protein spots were identified whose expression was different between control and ascorbic acid treatment groups.

***Keywords*—**Ascorbic acid, Proteomic analysis, S-180 implanted BALB/C mouse

I. Introduction

ASCORBIC acid (also termed ascorbate) is toxic to cancer cells when given intravenously at high concentrations [1]. Increasing evidence also indicates that ascorbic acid is selectively toxic to some types of tumor cells as a pro-oxidant, rather than as an anti-oxidant [2, 3]. At concentrations of 10 nM–1 mM, ascorbic acid induces apoptosis in neuroblastoma and melanoma cells [4, 5]. Furthermore, ascorbic acid modulates mouse myeloma cell growth in vitro, as well as modulating leukemic progenitor cell growth in cells from patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) [6-8]. The common characteristics of cancer cells are invasion and metastasis, both of which play important roles in secondary tumor development and progression, and which influence patient mortality. Attachment and penetration of cancer cells affects the extent of invasion and metastasis [9]. Cancer cells form tumors and spread by degrading the extracellular matrix (ECM) through various matrix metalloproteinases (MMPs) [10]. Nutrients such as lysine and ascorbic acid are postulated to act as natural inhibitors of ECM proteolysis, and as such have the potential to inhibit tumor growth and expansion [11]. These nutrients may exert their antitumor effects via inhibition of MMPs and strengthening the connective tissue surrounding cancer cells (a 'tumor encapsulating' effect). Additionally, it has been suggested that, through inhibition of hyaluronidase, ascorbic acid can prevent metastases by preventing degradation of the ground substance surrounding the tumor [12]. Different mechanisms are involved in the metastatic cascade including angiogenesis, cellular adhesion, local proteolysis, and tumor cell migration [9, 13]. Tumor development and progression consist of a series of complex processes involving multiple changes in gene expression [10]. The results of several experimental studies have shown that ascorbic acid inhibits tumor growth and metastasis [14]. Time-lapse analyses of Walker 256 carcinosarcoma cell migration showed that both the speed of movement and cell displacement are inhibited by ascorbic acid [14]. These results demonstrate that intact, unmodified ascorbic acid applied in physiologically relevant and nontoxic concentrations exerts an inhibitory effect on the migration of WC 256 carcinosarcoma cells, and that this may be one of the factors responsible for the anti-metastatic activity of ascorbic acid. Additionally, sodium ascorbate supplementation of drinking water inhibits subcutaneous tumor growth, enhanced levodopa methylester chemotherapy, and increased survival of B 16 melanoma-bearing mice [15]. Spontaneous metastasis was found to be inhibited by ascorbate in mice fed the restricted diet [15]. Recently, the ability of orally administered vitamins C and K3 to inhibit the development of metastases of mouse liver tumor (TLT) cells that have been implanted into the thigh of C3H mice was evaluated [16].We have observed that the rates of tumor formation decrease and the survival rates of the mice for 30 days increase after administration of high doses of ascorbic acid to mice implanted with S-180 cancer cells. To understand the molecular basis of ascorbic acid effect on tumor formation, we investigated the expression of proteins whose expression was altered in the presence of ascorbic acid. There has been no information on the protein products produced in tissues exposed to ascorbic acid, although numerous studies have examined the anti-cancer effect of ascorbic acid. In the present study, we performed two-dimensional gel electrophoresis (2-DE) using tumor tissues of mice implanted with S-180 cancer cells following the administration of a high dose of ascorbic acid.

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II. Procedure

A. Implantation of cancer cells and assessment of tumor formation

Viable neoplastic S-180 sarcoma cells (10^6) were implanted in mice via intraperitoneal injection. S-180 cell line was a mouse cell line originated from Swiss Webster Sarcoma180 tissue. The cells were cultured in vitro in RPMI 1640 medium

(Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Salt Lake City), 100 IU/mL penicillin, and 100 μg/mL streptomycin. The cells were subcultured at a split ratio (1:4) for injection; >95% of the total cells populations were viable as determined by the exclusion of trypan blue. The tumor formation rate was calculated according to the percent of the mice injected presenting with tumors. Mice in groups B, D, and E were intraperitoneally injected with ascorbate (1.5 mg/g body weight) every three days. The injections were performed by Huons Co. (Seoul, Korea). Body diameters were measured every five days once tumors were palpable. All mice were sacrificed by general anesthesia when spontaneous mortality appeared 30 days after tumor transplantation. The 30-day survival rate was determined for each group. For group C, D, and E mice, this survival rate represented surviving mice out of those in whom tumors had become established. After sacrifice, a detailed general autopsy of each mouse was performed to identify tumor formation. Primary tumors and organs or tissues suspected of harboring tumor such as liver and kidneys were macroscopically examined. Samples of liver hepatocytes and kidneys were taken for detailed histological examination. These samples were fixed in 10% neutral formalin, embedded in paraffin, cut into 5–7 μm thick sections using a microtome, and stained with hematoxylin and eosin.

B. Preparation of total protein extracts

To obtain total protein extracts, liver tissue was homogenized in 2-DE sample buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 100 mM dithiothreitol (DTT), and a protease inhibitor cocktail (Roche, Basel, Switzerland). Tissue was disrupted by five strokes with a sonicator. After 30 min incubation with DNase (100 U/mL) at 4°C, tissue lysates were centrifuged at 45,000 rpm for 45 min at 4°C. The supernatant was collected in a new tube.

C. Protein purification

Precipitation using trichloroacetic acid (TCA; Sigma-Aldrich, St. Louis, MO) and acetone was performed to purify protein. TCA (50% v/v) was added to produce a final concentration of 5%–8%. The sample was mixed by inversion and incubated on ice for 2 h. Following centrifugation at 14,000 rpm for 20 min the supernatant was discarded and the protein pellet was resuspended in 200 μL cold acetone. After incubation on ice for 15 min, the sample was centrifuged at 14,000 rpm for 20 min and dried. The dried pellet was dissolved in 2-DE sample buffer and the protein concentration was determined using a Bradford protein assay kit (Bio-Rad, Hercules, CA).

D. Isoelectric focusing (IEF)

Protein (800 μg) was diluted to a final volume of 300 μL in sample buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 100 mM DTT, and 0.5% carrier ampholyte (pH 4–7, Bio-Rad) and loaded on a 17 cm long gel with pH 4–7 gradient. A rehydrated immobilized pH gradient (IPG) strip was positioned gel side-down on the strip tray and covered with mineral oil. The voltage was sequentially increased from 100V–8000V to attain 80,000 total voltage h (300Vh at 100V, 400Vh at 200V, 1000Vh at 500V, 1000Vh at 1000V, 2000Vh at 2000V, 4000Vh at 4000V, for a total of 80000Vh at 8000V). During IEF, the temperature was set to 20°C. To solubilize the focused proteins, the IPGs were soaked in sodium dodecyl sulfate (SDS) equilibration buffer containing 6 M urea, 2%(w/v) SDS, 0.05 M Tris–HCl (pH 8.8), and 20% glycerol. The strip was treated with 10 mL of an equilibration solution containing 6 M urea, 2% (w/v) SDS, 0.05 M Tris–HCl (pH 8.8), 20% glycerol, and 20% DTT, and placed on a shaker for 10 min. Then strip was shaken in 10 mL of iodoacetamide (IAA) equilibration solution containing 6 M urea, 2%(w/v) SDS, 0.05 M Tris–HCl (pH 8.8), 20% glycerol, and 25% IAA for another 10 min. After briefly rinsing with 1x gel buffer, the IPG strip was loaded on the top of 12% SDS-polyacrylamide gel electrophoresis (PAGE) gel. Low melting point agarose consisting of 1% molten agarose solution with a trace amount of bromophenol blue was added. SDS-PAGE was performed for 30 min at a constant current of 16 mA and then at 24 mA at 4°C.

E. 2-DE gel image analysis

Electrophoretically separated proteins were visualized by Coomassie brilliant blue G-250 staining of the gels. Images were digitalized using a GS-800 calibrated densitometer (Bio-Rad) and analyzed by PD Quest 2-D software (Bio-Rad). Quantitative differences were determined only when a matched spot displayed the same degree of down- or up-regulation in duplicate experiments. Matching spots in gels from the same sample were identified and their intensities were measured using an Image Master 2-D system. Analysis was performed on approximate 170 different protein spots per sample. For each spot, the intensity value obtained in the ascorbic acid-treated gel was divided by that obtained in the control gel. The logs of these ratios (LR; the means and median values clustered around the 0 value) were then calculated. LR was expected if errors were associated with the analysis were random and normally distributed. Spots showing an expression 3.0-fold less or greater than the control were considered to represent a statistically significant differentially expressed protein species.

F. In-gel enzymatic digestion and mass spectrometry

Spots were excised from the stained gel, destained with 50 mM ammonium bicarbonate in 40% acetonitrile, and dried with a Speed Vac plus SC1 10 (Savant Holbook, HY). The excised spot was rehydrated in 10 ng/μL trypsin in 50 mM ammonium bicarbonate. After the rehydrated spot was placed on ice for 45 min and treated with 50 mM ammonium bicarbonate (10 μL), it was incubated at 37°C for 12 h.

G. Matrix assisted laser-desorption ionization time-of-flight tandem mass spectroscopy (MALDI TOF-MS/MS)

Digested samples were removed and subjected to a desalting/concentration step on a mZipTipC18 column

(Millipore, Billerica, MA) using acetonitrile as an eluent before MALDI-TOF -MS/MS analysis. Peptide mixtures were loaded on the MALDI system using the dried-droplet technique and α-cyano-4-hydroxycinnamic acid (Sigma) as matrix, and were analyzed using a 4700 Reflector spec #1 mass spectrometer (Applied Biosystems, Framingham, MA). Internal mass calibration was performed using peptides derived from enzyme autoproteolysis. The Data explorer software package (Applied Biosystems) was used to identify spots from the ProFound database by mass searching all taxa sequences. Candidates identified by peptide mapping analysis were evaluated further by comparing their calculated masses and isoelectric points using the experimental values obtained by 2-DE.

III. Results

A. Influence of ascorbate on tumor formation and survival

In mice injected with S-180 tumor cells, ascorbic acid treatment inhibited tumor formation during the treatment period (Fig. 1). The tumor then began to grow and caused ascites. Survival of mice sensitive to ascorbate (groups D and E) was more than twice that of the survival of mice in group C. Due to ascites, the mean body width of group C mice was larger than that of ascorbate-treated mice in groups D and E . Soon after tumor injection, ascorbic acid treatment produced a distinct inhibition of solidification and ascites. Mortality was accelerated by tumor necrosis, ulcerations, and infections. Histological examinations were performed on some of the dead mice. The examined primary tumors were voluminous and often exhibited large ulcerations and subsequent dissemination of their contents with macroscopically detectable metastases. We next investigated whether there was any difference in tumor metastases between the control and experimental groups of mice. Macroscopic and microscopic examinations of serial liver sections revealed a distinct difference in the number of mice bearing liver metastases between the control and the ascorbic acid-treated groups of mice. Detailed autopsies and microscopic examinations showed metastases in the hepatocytes, which displayed an eosinophilic cytoplasm with round nuclei. These were contained within in the liver hepatocytes rather than being present on the stroma of the liver. In the experimental group, seven of 31 mice (23%) exhibited liver metastases, while 12 of 30 control mice (40%) possessed liver metastases, showing a distinct and significant (p=0.034) reduction in the ascorbic acid-treated group as compared with the control group. Detailed autopsies and microscopic examinations did not demonstrate metastases in any kidney.

B. Changes in liver tissue proteome profile after ascorbic acid treatment

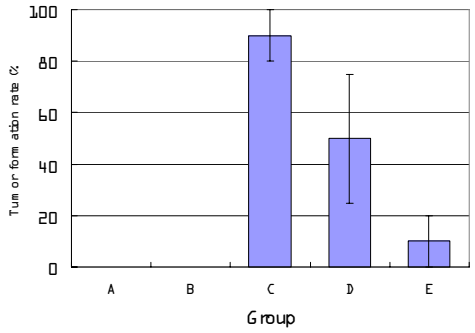

Fig. 1. Effect of ascorbic acid on tumor formation and survival rate. Thirty-day survival rate was determined within each group. In case of groups C, D, and E, the survival rate represented survival of tumor-implanted mice. The values were obtained from eight independent experiments. In each experiment, each group was composed of 10 mice. Data represent the mean ± SD. Asterisk (*) indicates P<0.05 compared to control group C (Student's t test).

To investigate the alterations associated with liver metastasis on a molecular basis, proteomic differential display analysis for the expression of proteins in the tumor tissue of mice in groups treated with or without ascorbic acid was performed by 2-D gel electrophoresis and MALDI-TOF MS/MS. Protein expression

TABLE I
PROTEIN DESCRIPTION

	APOLIPOPROTEIN A-
	GROWTH FACTOR RECEPTOR BOUND PROTEIN 2
	UNNAMED PROTEIN PRODUCT
	UBIQUITIN-CONJUGATING ENZYME E2-25K
	MYELOID BACTENECIN
	ACTIN RELATED PROTEIN 2/3 COMPLEX, SUBUNIT 5
	MITOCHONDRIAL RIBOSOMAL PROTEIN S6
	PEROXIREDOXIN 3
	RHO, GDP DISSOCIATION INHIBITOR (GDI) BETA
	TUMOR PROTEIN, TRANSLATIONLLY-CONTROLLED 1
	ACIDIC RIBOSOMAL PHOSPHOPROTEIN P0
	VIMENTIN
	EUKARYOTIC TRANSLATION INITIATION FACTOR 3, SUBUNIT
	SET TRANSLOCATION
	CAPPING PROTEIN (ACTIN FILAMENT) MUSCLE Z-LINE, BETA ISOFORM B
	LATEXIN
	ATP SYNTHASE, H+ TRANSPORTING, MITOCHONDRIAL F0 COMPLEX, SUBUNIT D
	A CHAIN A, RECOMBINANT MOUSE L-CHAIN FERRITIN
	FRIL1_MOUSE FERRITIN LIGHT CHAIN 1 (FERRITIN L SUBUNIT 1)
	FERRITIN HEAVY CHAIN 1

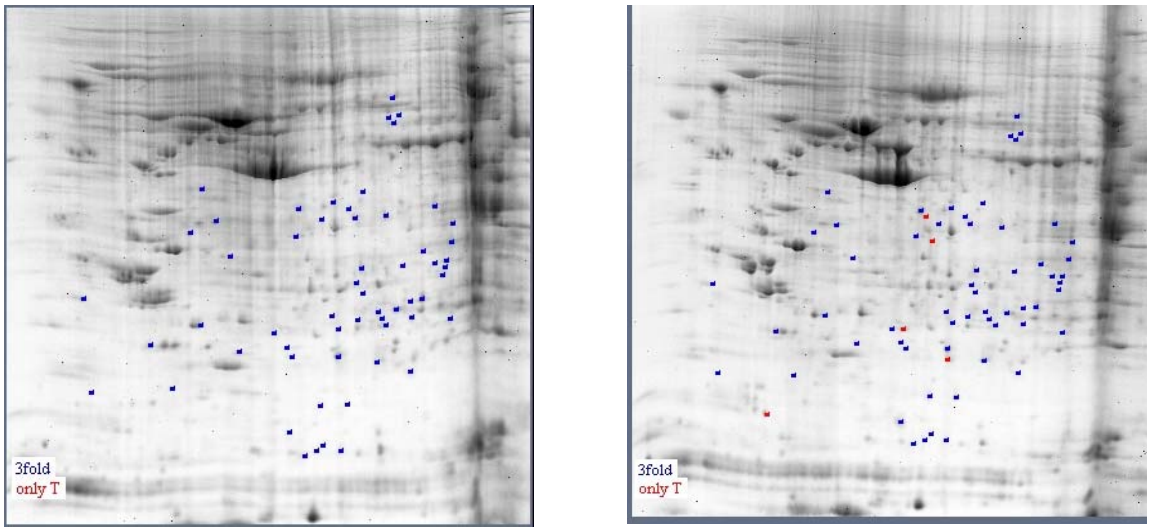

Fig. 2. Examination of liver proteins by 2-DE. Results shown are representative of four independent experiments. The images were analyzed by ImageMaster 2D software and proteins were identified by MALDI-TOF MS/MS. Total protein extracts were analyzed by 2DE and gels were stained with Coomassie brilliant blue G-250. Vertical axes represent apparent molecular mass (kDa) and horizontal axes pH values. Acquired images of four independent experiments showed repetitive pattern.

was assessed in six samples each from each group obtained on the same schedule under the same conditions. More than 150 protein spots were visualized on the 2-DE gels, and the differences in spot intensities between groups treated with or without ascorbic acid were compared visually and analyzed for each gel. Expression comparisons of each Coomassie brilliant blue-stained spot are shown in Fig. 2. Twenty protein spots whose expression differed between groups treated with or without ascorbic acid were apparent as shown in Table 1.

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