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Inhibition of Angiogenesis-driven Kaposi's Sarcoma Tumor Growth in Nude Mice by Oral *N*-Acetylcysteine[1]

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ABSTRACT

The thiol *N*-acetyl-L-cysteine (NAC), an analogue and precursor of reduced glutathione, has cancer chemopreventive properties attributable to its nucleophilicity, antioxidant activity, and a variety of other mechanisms. We demonstrated recently that NAC has anti-invasive, antimetastatic, and antiangiogenic effects in *in vitro* and *in vivo* test systems. In the present study, s.c. transplantation of KS-Imm cells in (CD-1)BR nude mice resulted in the local growth of Kaposi's sarcoma, a highly vascularized human tumor. The daily administration of NAC with drinking water, initiated after the tumor mass had become established and detectable, produced a sharp inhibition of tumor growth, with regression of tumors in half of the treated mice along with a markedly prolonged median survival time. The production of vascular endothelial growth factor (VEGF) and certain proliferation markers (proliferating cell nuclear antigen and Ki-67) were significantly lower in Kaposi's sarcomas from NAC-treated mice than from control mice. Treatment of KS-Imm cells with NAC *in vitro* resulted in a dose-dependent inhibition of chemotaxis and invasion through inhibition of gelatinase-A (matrix metalloproteinase-2, MMP-2) activity without altering MMP-2 or MMP-9 mRNA levels. NAC also significantly inhibited VEGF production but did not affect proliferation markers *in vitro*. Reverse transcription-PCR analysis indicated that total VEGF mRNAs were reduced by 10 mM NAC. Taken together, these findings provide evidence that NAC, the safety of which even at high doses has been established in almost 40 years of clinical use, in addition to its chemopreventive action, has a strong antiangiogenic potential that could be exploited for preventing cancer progression as well as used in cancer adjuvant therapy.

INTRODUCTION

The growth of most solid tumors beyond dimensions of a few mm^3 appears to require vascularization of the expanding tumor mass (1–3). Inhibition of tumor angiogenesis may limit tumor growth and is currently under extensive investigation as a potential approach to cancer progression prevention and for cancer therapy (2). During neovascularization, in response to an angiogenic stimulus, endothelial cells lose contact inhibition, proliferate, migrate, and invade toward the stimulus and eventually differentiate to organize new vessels. A local balance between angiogenesis stimulatory and inhibitory signals appears to regulate the angiogenic process (3, 4). A major stimulatory factor is VEGF,[3] a highly specific mitogen for vascular endothelial cells that plays a key role in induction of angiogenesis (reviewed in Ref. 5). VEGF is expressed in spatial and temporal association with the physiological events of angiogenesis *in vivo*. Inhibition of VEGF activity by neutralizing antibodies (6) or by the introduction of dominant-negative VEGF receptors into endothelial cells of tumor-associated blood vessels (7) resulted in inhibition of tumor growth and in tumor regression, indicating that VEGF is a major initiator of tumor angiogenesis. These observations have sparked intense efforts directed at the development of efficient inhibitors of VEGF production and VEGF signal transduction for use in tumor growth control (5).

KS is a highly vascularized tumor associated with infection by the KSHV/HHV8 herpesvirus (8). It is classified into four epidemiological forms: sporadic (rare in the elderly); epidemic (AIDS associated); iatrogenic (posttransplant associated); and endemic (in some areas of sub-Saharan Africa). All of the KS forms show a clear predominance in males, with an ~4-fold higher risk in males as opposed to females among HHV8-seropositive subjects (9). The main histological features of KS are spindle cells, an inflammatory infiltrate, and the formation of a dense, poorly organized capillary network recruited from the host (10). KS spindle cells release a mixture of potent stimulators of endothelial cell migration, invasion (11–14), and gelatinase production *in vitro* (15), which correlates with a powerful induction of angiogenesis *in vivo* (14). We have isolated in our laboratory an immortalized KS cell line (KS-Imm; Ref. 16) that has the general characteristics of KS spindle cells that produce VEGFs and express functional VEGF receptors (17–20) and, when injected s.c. into nude mice, produce large, highly vascularized tumors (21). This *in vitro* and *in vivo* KS-Imm system provides an excellent model for identification of potential angiogenesis inhibitors and for testing their effectiveness in inhibition of tumor growth *in vivo*.

Modulation of extracellular and intracellular thiols has been used as a chemopreventive approach that is currently being investigated as a novel strategy in cancer prevention. One of the most extensively studied thiols is NAC, a cytoprotective drug with multiple preventive properties (22). The protective effects of NAC in carcinogenesis have been shown to depend largely on the antigenotoxic activity associated with its nucleophilicity and antioxidant properties along with a variety of other mechanisms (23, 24). Many of the effects of NAC are associated with its ability to act extracellularly as an analogue of GSH and intracellularly as a precursor of cysteine and GSH (23, 24). In addition to these effects, it appears that NAC can hinder the carcinogenic process at other steps as well. NAC has been shown to inhibit tumor cell invasion *in vitro* and metastasis formation *in vivo*, apparently through inhibition of secreted matrix metalloproteinase activity (25). NAC was also found to inhibit initial tumor take (25), and recent studies have indicated that these effects may be attributable to inhibition of angiogenesis by NAC (26), again apparently through inhibition of metalloproteinases.

The observation that NAC may have an antiangiogenic activity suggested that it may also be able to inhibit further growth and expansion of established tumors that depend on induction of vascu-

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[3] The abbreviations used are: VEGF, vascular endothelial growth factor; KS, Kaposi's sarcoma; NAC, *N*-acetyl-L-cysteine; GSH, reduced glutathione; SFM, serum-free medium; CM, conditioned medium; RT-PCR, reverse transcription-PCR; MMP, matrix metalloprotease; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCNA, proliferating cell nuclear antigen; LI, labeling index; KSHV/HHV8, KS-associated herpesvirus/human herpesvirus 8; HAART, highly active antiretroviral therapy; ROS, reactive oxygen species.

larization. We therefore tested the activity of NAC on KS-Imm cells. NAC inhibited KS-Imm cell invasion and chemotaxis *in vitro*, and orally delivered NAC significantly inhibited the growth of KS tumors *in vivo*, which was associated with improved survival of treated mice. In addition to inhibition of gelatinase activity, NAC appeared to directly affect the production of VEGF by the KS-Imm cells, resulting in reduced angiogenesis and restricted tumor growth attributable to interference with paracrine and autocrine loops. These data suggest that NAC may be useful in the prevention of progression and therapy of KS and possibly other angiogenic tumors.

MATERIALS AND METHODS

Cells. The cell line KS-Imm was isolated in our laboratory from a kidney-transplanted, immunosuppressed patient, as described previously (16). KS-Imm cells were grown in RPMI 1640 containing 10% FCS and 1% glutamine. For the *in vivo* experiments, the cells, at 80% confluence, were harvested by trypsinization, counted, and resuspended in SFM.

Animals and Treatments. The effect of NAC on the KS growth *in vivo* was tested by using 12 male and 12 female (CD-1)BR nude mice (Charles River, Calco, Lecco, Italy), 7 weeks of age, having an average weight of ~25 g. The animals were housed in pathogen-free conditions. Each mouse was injected s.c. in the flank region with 5×10^6 KS-Imm cells mixed with liquid Matrigel to a final volume of 250 μl/nude mice. Thirteen days after tumor cell injection, when a distinct tumor mass was detectable in all animals, they were randomized into two groups of male mice and two groups of female mice with the same average tumor size. At this point, one group received NAC daily in the drinking water at a dose of 2 g/kg body weight (based on previous observations that nude mice drink consistently 4 ml of water/day under our housing conditions, the NAC concentration was adjusted to average body weight of each cage), while the other group received NAC-free drinking water. The animals were weighed, and the two major diameters of tumors were measured every 2–3 days by using a caliper to estimate the tumor volume by standard calculations. This experiment was terminated after 31 days. In an additional experiment, we assessed the effects of NAC treatment on the survival of tumor-bearing nude mice. Twelve male nude mice received injections of KS-Imm cells, as described above. Distinct tumors formed in all animals after 6–8 days. On day 10, the animals were randomized into two groups with the same average tumor size; 6 continued to receive drinking water alone; and 6 were given water with NAC as described above. Tumor growth was assessed as above, and the survival times were recorded.

Housing and all treatments of animals were in accordance with the national and European Community guidelines (D.L. 2711/92 No. 116; 86/609/EEC Directive).

Chemotaxis and Chemoinvasion Assays. Chemotaxis and chemoinvasion assays were performed with KS-Imm cells in Boyden chambers as described previously (27, 28). Briefly, 1.5×10^5 cells in SFM with 0.1% BSA were placed in the upper compartment without NAC or with either 0.1, 1, or 10 mM NAC. The two compartments of the Boyden chamber were separated by 8-μm pore-size polycarbonate filters coated with 50 μl of 5 μg/ml gelatin (Sigma Chemical Co., St. Louis, MO) for the chemotaxis assay or with Matrigel (15 μg/ml), a reconstituted basement membrane, for the invasion assay. Supernatants from NIH3T3 cells (NIH3T3-CM) were used as chemoattractants in the lower chamber. After incubation for 6 h at 37°C in 5% CO_2, the filters were recovered, the cells on the upper surface were mechanically removed, and those on the lower surface were fixed and stained. The migrated cells were counted in five to ten fields for each filter under a microscope. The experiments were performed in quadruplicate and repeated twice.

Gelatin Zymography. KS-Imm cells were incubated in serum-free medium overnight without or with NAC or vitamin C (ascorbic acid) at the concentrations indicated. The supernatants were collected, and gelatin zymographs were then performed as described previously (25). Briefly, SDS-PAGE gels were prepared containing copolymerized gelatin at a final concentration of 0.6 mg/ml. Enzyme-containing samples were redissolved in 40 mM Tris (pH 7.5) and electrophoresed. After electrophoresis, the gels were washed in 2.5% Triton X-100 for 30 min to remove SDS and incubated for 18 h at 37°C in collagenase buffer (40 mM Tris, 200 mM NaCl, and 10 mM $CaCl_2$, pH 7.5). In other experiments, KS-Imm cells were incubated in SFM for 24 h, the supernatant was collected, concentrated, and loaded onto a single, wide lane. After electrophoresis, the single lane was divided into four equal strips and incubated, as indicated, in either collagenase buffer alone or in collagenase buffer containing 0.1, 1, or 10 mM NAC or with 0.1 or 1 mM vitamin C. The gels were then stained in 0.1% Coomassie brilliant blue and destained. Enzyme-digested regions were observed as white bands against a blue background.

Analysis of mRNA Expression. Total RNA was isolated from control and treated cells using the TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. Reverse transcription with oligo dT primers and semiquantitative PCR were performed as described (29). Images relative to results that satisfied linearity criteria were subjected to semiquantitative analysis.

A multiplex approach was used for determination of levels of proteins associated with VEGF signaling, in particular total VEGF, based on a commercial kit (VEGF MPCR Set-2; Maxim Biotech, Inc.). cDNA was synthesized as above and amplified with 32 cycles of 94–60-72°C for 30 s each. The resulting DNA was run on a 2% agarose gel and stained with ethidium bromide, and the image was captured with a Gel-Doc (Bio-Rad) imager. The image was analyzed, and samples were normalized to the expression of GAPDH similar to above.

VEGF Immunocytochemistry and Cell Proliferation Assessment in Cultured KS-Imm Cells. The cells were harvested and suspended in medium with either 0, 0.1, 1, or 10 mM NAC and then plated and incubated for 24 h. Alternatively, the cells were allowed to adhere for 6 h prior to addition of NAC and incubated with NAC for an additional 18 h. In both cases, after incubation the cells were harvested, suspended, and spun onto slides by means of a cytocentrifuge followed by fixation in absolute methanol for 5 min. VEGF immunoreactivity was evaluated using a rabbit polyclonal antibody raised against a peptide mapping to the NH_2 terminus of human VEGF (VEGF A-20 rabbit IgG; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 1:100 dilution. Immunohistochemical staining was performed using a Rabbit Extravidin Peroxidase Staining kit (Sigma Chemical Co.), following the manufacturer's instructions and using 3-amino-9-ethylcarbazole as a chromogen.

PCNA and Ki-67 immunoreactivities were detected by using the NCL-PCNA and the NCL-Ki-67 kits (Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom), respectively, following the manufacturer's instructions. These kits use monoclonal antibodies (PCNA, clone PC10; Ki-67, clone MM1) and the avidin/biotinylated horseradish peroxidase complex technology (ABC technique). Slides were scored at ×400, and 1000 cells/slide were examined in each one of three separate experiments. The number of positive-stained cells was recorded and expressed as a percentage of the total number of cells (LI). Immunohistochemical staining for factor VIII was performed as described previously (30).

VEGF Immunohistochemistry and Cell Proliferation Assessment in Tumor Sections. Tumors were processed according to routine histopathology techniques (formalin fixation and paraffin embedding). Tumor sections (5 μm) were cut and placed onto slides treated with poly-L-lysine (Poly-Prep Slides; Sigma Chemical Co. Diagnostics, St. Louis, MO). VEGF immunoreactivity and immunohistochemical staining were performed as described above. Slides were scored at ×200; 10 fields/slide were examined, and the average number of VEGF-positive foci per microscopic field was recorded.

PCNA and Ki-67 immunoreactivities were detected as described above. Slides were scored at ×400, and 1000 cells/slide were examined. The number of positive-stained cells was recorded and expressed as a percentage of the total number of cells (LI).

VEGF Quantification by ELISA. VEGF protein released into the conditioned media of KS-Imm cells was measured using a commercial ELISA kit for VEGF (LISTARFISH; CYTImmune Sciences, College Park, MD) following the manufacturer's instructions. The supernatants were collected after 24 h of incubation with 0.1, 1, or 10 mM NAC or with 0.1 or 1 mM vitamin C in SFM. The values obtained for the CM samples were compared with a calibration curve prepared by testing serial dilutions of VEGF standard. The assay was run in triplicate and repeated twice with similar results; data of the two experiments were pooled for statistical analysis.

Statistical Analyses. Correlations between NAC doses and effects were evaluated by using Spearman's and simple regression tests. Comparisons of mean data were made by Student's *t* test for unpaired data, and comparisons of tumor growth curves as related to treatment were made by ANOVA. Survival data (Kaplan-Meier plots) were analyzed by the log-rank (Mantel-Cox) test.

RESULTS

Effect of NAC on KS-Imm Migration, Invasion, and Gelatinolytic Activity *in Vitro*. The invasive and chemotactic activities of KS-Imm cells were assessed in Boyden chambers in the presence and in the absence of Matrigel coating, respectively. KS-Imm cells strongly migrated and invaded in response to NIH3T3-CM, whereas the response in the absence of a chemoattractant (SFM) was limited (data not shown). Similar to that observed with other tumor cell types, the addition of NAC to the KS-Imm cells induced a dose-dependent inhibition of invasion that was statistically significant at 1 and 10 mM ($P < 0.02$). NAC also inhibited the chemotaxis of KS-Imm cells, with strong (60%) and significant ($P < 0.0001$) inhibition at 10 mM. Previous studies indicated that NAC inhibition of tumor cell invasion correlated with inhibition of MMPs. To test the effect of NAC on the MMP gelatinolytic activity released by KS-Imm cells, we analyzed the supernatants of KS-Imm cells treated with increasing concentrations of NAC. The controls showed characteristic gelatinase activities corresponding to the M_r 72,000 gelatinase-A (MMP-2) and, with less activity, the M_r 92,000 gelatinase-B (MMP-9). NAC-treated cells showed a dose-dependent decrease in the active gelatinases released (Fig. 1*a*), with near complete suppression of active gelatinase release at 25 mM. We then examined whether NAC can directly affect enzymatic activity, by inclusion of increasing concentrations of NAC in the collagenase buffer of identical strips of a single electrophoresis of untreated KS-Imm cell supernatants. These data confirmed that this drug dose dependently inhibited the enzymatic activity of both MMP-2 and MMP-9, with complete inhibition at 10 mM (Fig. 1*b*).

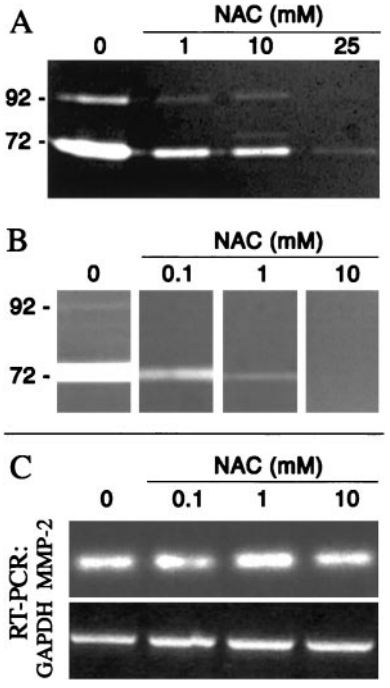

Fig. 1. Effect of NAC on the release and activity of the gelatinases MMP-2 (M_r 72,000) and MMP-9 (M_r 92,000) by KS-Imm cells. *A*, supernatants of the cells incubated for 24 h in serum-free medium with or without varying concentrations of NAC as indicated. The supernatants were collected and analyzed by gelatin zymography at equivalent protein concentration. The enzyme digested regions were observed as white bands against a blue background. *B*, a supernatant of KS-Imm cells incubated in SFM for 24 h was loaded onto a single, wide zymography gel electrophoresis lane. After electrophoresis, the lane was divided into four equal parts and incubated, as indicated, in either collagenase buffer alone or in collagenase buffer containing 0.1, 1, or 10 mM NAC. NAC dose dependently inhibited the enzymatic activity present in the gel. *C*, KS-Imm cells were incubated 24 h in the absence or presence of either 0.1, 1, or 10 mM NAC. Total RNA was extracted, and mRNA levels for MMP-2 were determined, relative to that of GAPDH, by semiquantitative RT-PCR.

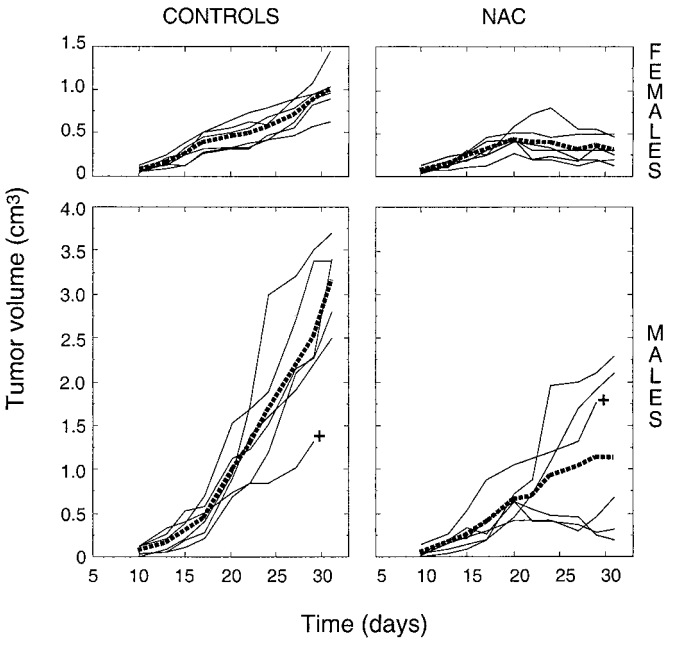

Fig. 2. Growth of KS mass in 12 male and 12 female (CD-1)BR nude mice receiving, at time 0 of the experiment, a s.c. injection of KS-Imm cells (5×10^6 cells/mouse) in the flank region. Starting on day 13, when the tumor mass was already measurable, the mice were either untreated or treated daily with NAC (2 g/kg body weight) in drinking water until the end of the experiment (day 31). Each *curve* refers to the tumor growth in each one of the 24 mice, and the *bold dashed curves* are the means of the values measured within each group of 6 mice. The + symbols refer to the death times of two mice that died before the end of the experiment.

To investigate the possible effects of NAC on MMP-2 transcription, RNA was extracted from KS-Imm cells treated either with 0, 0.1, 1, or 10 mM NAC and analyzed by semiquantitative RT-PCR. Densitometric analysis of these bands and comparison with the intensity of the GAPDH signal as a standard indicated no significant variation of MMP-2 mRNA production upon NAC treatment (Fig. 1*c*). Another antioxidant molecule, vitamin C, did not affect the enzymatic activity of MMP-2 or MMP-9, nor did it alter mRNA expression for these molecules (data not shown).

Inhibition by NAC of Kaposi's Sarcoma Growth in Nude Mice. Tumor masses became detectable and measurable ~10 days after s.c. injection of KS-Imm cells in the flank region of nude mice. On day 13, these animals were randomized into groups of similar tumor volume and NAC treatment was started. At that time, the average tumor volume (mean ± SD) was 0.15 ± 0.06 cm^3 in the group of control male mice, and 0.14 ± 0.06 cm^3 in the group of male mice selected for NAC administration. For the female mice, the average tumor volume was 0.16 ± 0.12 cm^3 in the control group and 0.15 ± 0.08 cm^3 in the group to receive NAC.

As shown in Fig. 2, the tumor volume progressively grew in each one of the control mice. After 31 days, when this experiment was terminated, the tumor volume was significantly ($P < 0.001$) higher in male (3.2 ± 0.5 cm^3) than in female (1.0 ± 0.3 cm^3) mice. NAC administration progressively inhibited tumor growth with time in both male and female animals; after 31 days, the average tumor volume was 1.1 ± 1.0 cm^3 in NAC-treated males and 0.3 ± 0.2 cm^3 in NAC-treated females. Comparison of tumor growth curves by ANOVA showed that in male mice the differences between controls and NAC-treated groups were close to statistical significance on days 20 ($P = 0.06$) and 24 ($P = 0.09$) and were significant on days 22 ($P = 0.018$), 26 ($P = 0.013$), 28 ($P = 0.02$), and 31 ($P = 0.004$). In females, the differences were statistically significant for all measure-

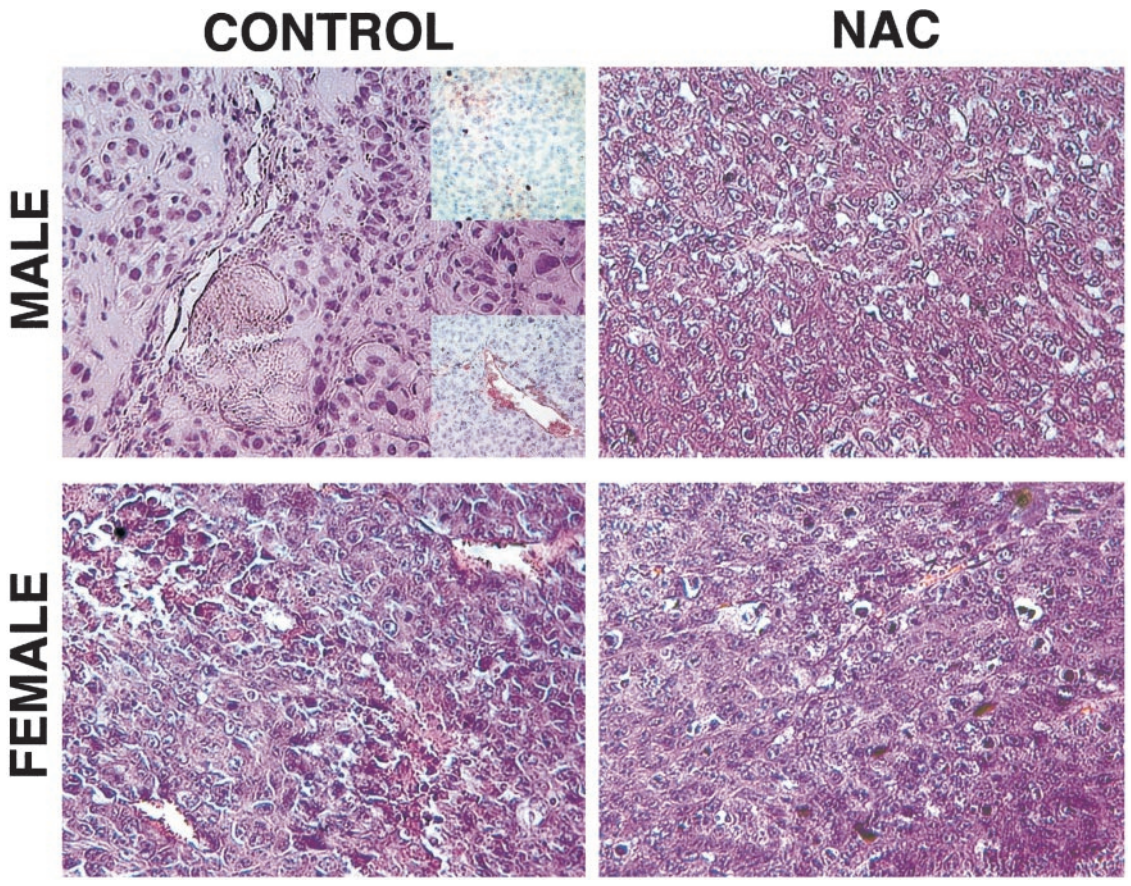

Fig. 3. Histology and immunohistochemistry of the KS-Imm tumors in male and female, control and NAC-treated mice, as indicated. The large panels are H&E-stained paraffin sections (nominal magnification, ×200); large vessels are readily visible in the controls. *Lower inset*, immunohistochemical staining for factor VIII; the cells lining the vessels are stained. *Upper inset*, immunohistochemical staining for VEGF protein; foci of cells expressing VEGF in a punctate pattern are evident.

ments made after day 27, specifically on days 27 ($P = 0.004$), 29 ($P = 0.0002$), and 31 ($P = 0.0002$).

There was a marked interindividual variability and differential responsiveness of mice to treatment with NAC (Fig. 2). Interestingly, in half of the male animals, there was not only reduced tumor growth but an evident trend to regression of the tumor volume with NAC treatment. In the females, all tumors appeared to respond to NAC treatment, resulting in a progressive decrease in the mean volume of tumors from day 20 (0.4 ± 0.1 cm^3) to day 31 (0.3 ± 0.2 cm^3).

Histological analysis of the recovered tumors indicated the presence of numerous large, irregular vessels in the control tumors (Fig. 3) that were frequently lined by factor VIII-positive cells (*lower inset*). NAC-treated tumors showed fewer and smaller vessels in both males and females as compared with controls (Fig. 3). No notable differences were found between the tumors obtained from the male or the female mice within the control groups or the NAC-treated groups.

Enhancement by NAC of Survival Times in Nude Mice Bearing KS. An additional experiment was designed to assess not only the growth of KS tumors but also the survival of the tumor-bearing nude mice administered either NAC or water alone (controls). Twelve male mice were injected with KS-Imm cells as above; on day 10 when all animals showed distinct tumor masses, they were randomized into two groups of equal tumor volume. At this point, 6 animals received NAC in the drinking water as described above, and 6 were given water alone. The tumor volumes were 0.09 ± 0.04 cm^3 *versus* 0.11 ± 0.05 cm^3 on day 10 in control mice and in those randomly selected for NAC administration, respectively.

After initiation of NAC treatment, the curves indicating the mean volumes of tumors in the two groups (Fig. 4, *dashed bold curves*) became strongly divergent. On day 38, *i.e.*, the last day when all 12 animals were still alive, the tumor volume was 9.8 ± 6.2 cm^3 in control mice as opposed to 1.3 ± 1.1 cm^3 in NAC-treated mice. The difference between the two groups was close to the significance threshold on day 15 ($P = 0.06$) and became statistically significant at all subsequent measurements, *i.e.*, on days 17 ($P = 0.03$), 20 ($P = 0.03$), 22 ($P = 0.04$), 24 ($P = 0.03$), 27 ($P = 0.03$), 29 ($P = 0.03$), 31, 34, 36, and 38 (all $P = 0.02$). There were no significant alterations in the body weights of the control or NAC-treated animal groups over the entire course of the experiment.

The 6 control mice died on days 38, 38, 45, 45, 59, and 71, whereas NAC-treated mice died on days 79, 105, 108, 108, 111, and 114, with median survival times of 45 and 108 days, respectively. Kaplan-Meier plots of survival data (not shown), analyzed by log-rank (Mantel-Cox) test showed a significant difference between the two groups ($\chi^2 = 12.168$, $P = 0.0005$).

As observed in the previous experiment (Fig. 2), there was considerable interindividual variability in tumor growth patterns both in control mice and NAC-treated mice. In the NAC-treated mice, 4 of the 6 animals showed a regression of the neoplastic mass after 15–55 days, which in 3 cases appeared to be complete (Fig. 4). Of the remaining animals, 1 exhibited a very slow growth of the neoplastic mass, and the other showed an initial tumor growth similar to that of the slowest growing control mouse, which then expanded late in the experiment.

VEGF and Proliferation Markers in KS Transplanted into Nude Mice. VEGF and two proliferation markers (PCNA and Ki-67) were evaluated by immunohistochemical methods in the KS tumors of half of the 24 mice used in the experiment shown in Fig. 2. The

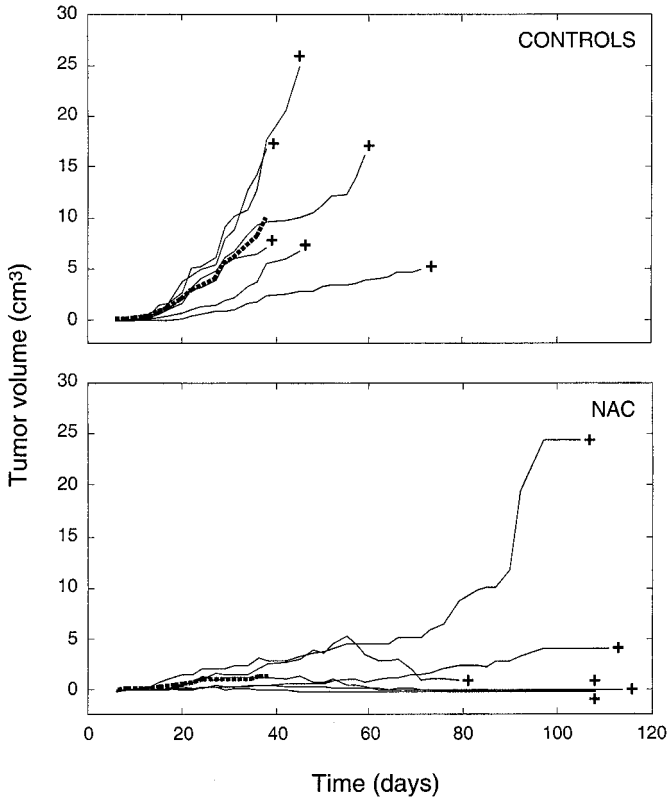

Fig. 4. Growth of KS tumors and animal survival in 12 male (CD-1) BR nude mice that received 5×10^6 KS-Imm cells s.c. in the flank region (time 0). After 10 days, when the tumor mass was already measurable, the mice were sorted into two groups either untreated (6) or treated daily with NAC (6) in the drinking water as above. Tumor growth was measured, and the animals were followed until "spontaneous" death. *Thin lines*, the tumor volume in individual mice over time; +, the time of death of each animal. *Bold dashed curves*, the means of the values measured within each group of 6 mice until the death of the first animal.

proportion of PCNA- or Ki-67-positive cells was expressed as LI, because the positive nuclei can be easily distinguished from negative nuclei. Conversely, the results for VEGF were expressed as number of positive foci/microscopic field, because this protein is localized in the cytoplasm and the cytoplasmic borders are not clearly visible in tissue sections. Moreover, VEGF was not evenly distributed in each tumor section but appeared in the form of diffuse foci of positive cells (Fig. 3, *upper inset*) histologically characterized as KS tumor cells. The results of these analyses are summarized in Table 1. The number of VEGF-expressing tumor cell foci and the frequency of the two proliferation markers (PCNA and Ki-67) were significantly decreased in NAC-treated mice as compared with controls.

When evaluating all 12 mice, there was a high and significant correlation between tumor volume and either PCNA ($r = 0.879$; $P < 0.0001$), Ki-67 ($r = 0.843$, $P < 0.0001$), or VEGF ($r = 0.722$, $P < 0.008$). Despite the small size of the subgroups, the significance of the correlation between volumes and either PCNA, Ki-67, or VEGF expression was maintained within the following subgroups: 6 control mice, irrespective of their gender ($r = 0.937$, $P = 0.002$ for PCNA; $r = 0.935$, $P = 0.02$ for Ki-67; $r = 0.898$, $P = 0.06$ for VEGF); 6 NAC-treated mice, irrespective of their gender ($r = 0.839$, $P = 0.002$ for PCNA; $r = 0.764$, $P = 0.05$ for Ki-67; $r = 0.937$, $P = 0.002$ for VEGF); 6 male mice, irrespective of treatment ($r = 0.950$, $P = 0.001$ for PCNA; $r = 0.954$, $P = 0.0008$ for Ki-67; $r = 0.928$, $P = 0.0025$ for VEGF); and 6 female mice, irrespective of treatment ($r = 0.928$, $P = 0.0025$ for PCNA; $r = 0.941$, $P = 0.002$ for Ki-67; $r = 0.954$, $P = 0.0009$ for VEGF).

VEGF and Proliferation Markers in Cultured KS Cells Treated with NAC. To confirm the specific effects of NAC on the monitored biomarkers *in vivo*, we evaluated modulation of the same parameters under controlled conditions in cultured KS cells. VEGF expression in KS-Imm cells was evaluated by immunohistochemical methods. NAC, added to cell suspensions at plating and kept for 24 h, inhibited VEGF expression in a concentration-related fashion, with significant inhibition at 1 and 10 mM (Table 2). Fig. 5 shows as an example the appearance of KS-Imm cells processed by immunocytochemical staining for the detection of human VEGF. The protein, expressed in a granular pattern, is well evident in the cell cytoplasm (Fig. 5),

Table 2 *Immunocytochemistry LI for VEGF, PCNA, and Ki-67 in KS-Imm cells treated with varying concentrations of NAC at the time of plating*

NAC (mM)	LI (%)		
	VEGF	PCNA	Ki-67
0	19.3 ± 5.1[a]	97.9 ± 3.2	86.7 ± 4.0
0.1	18.3 ± 3.8	97.0 ± 2.6	85.0 ± 3.6
1	9.0 ± 2.6[b]	98.0 ± 2.0	88.0 ± 4.6
10	0.7 ± 1.2[c]	97.0 ± 2.6	85.7 ± 4.5

[a] All results are mean ± SD of three separate experiments.
[b] $P < 0.05$, compared with NAC-free controls.
[c] $P < 0.01$, compared with NAC-free controls.

Table 1 *Immunohistochemistry LI for PCNA and Ki-67 and foci of VEGF-positive cells as related to gender, treatment, and tumor volume in KS-transplanted (CD-1)BR nude mice*

Gender	Treatment	Animal code	Tumor volume (cm^3)	LI (%)		Foci of VEGF-positive cells/microscopic field
				PCNA	Ki-67	
Male	Control	1	2.8	41.7	22.6	3.3
		2	3.4	45.8	24.5	2.7
		3	1.7	40.4	21.0	3.9
Mean ± SD			2.6 ± 0.9	42.6 ± 2.8	22.7 ± 1.8	3.3 ± 0.6
Male	NAC	7	1.8	28.4	11.0	2.5
		8	0.7	29.3	13.5	1.0
		9	0.3	15.9	8.5	0.8
Mean ± SD			0.9 ± 0.8	24.5 ± 7.5[a]	11.0 ± 2.5[a]	1.4 ± 0.9[b]
Female	Control	13	1.4	37.4	20.9	2.9
		14	0.9	23.2	14.5	1.6
		15	0.6	31.5	16.5	2.3
Mean ± SD			1.0 ± 0.4	30.0 ± 7.1	17.3 ± 3.3	2.3 ± 0.7
Female	NAC	19	0.3	19.2	10.4	0.9
		20	0.3	20.4	12.1	1.2
		21	0.2	16.5	7.7	1.0
Mean ± SD			0.3 ± 0.1[b]	18.7 ± 2.0[b]	10.1 ± 2.2[b]	1.0 ± 0.2[b]

[a] $P < 0.01$, compared with control.
[b] $P < 0.05$, compared with control.

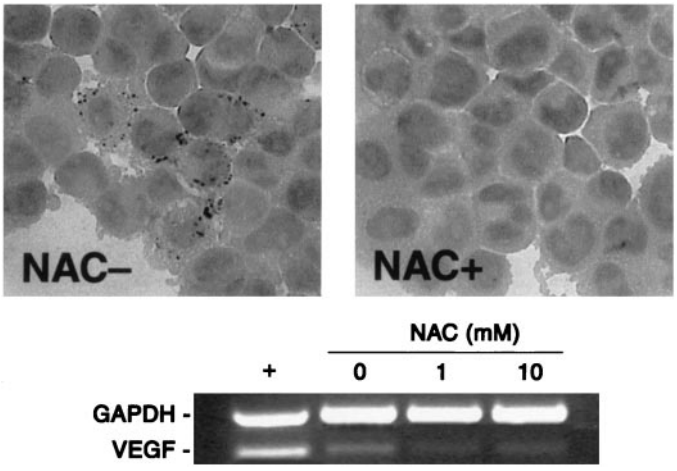

Fig. 5. The effects of NAC on VEGF expression *in vitro*. *Upper panels*, immunocytochemical detection of human VEGF in cultured KS-Imm cells *in vitro*. The VEGF staining appears as *dark grains* in the cytoplasm of KS-Imm cells. Few grains are evident in the NAC treated samples. ×1000. *Lower panel*, semiquantitative multiplex RT-PCR for an internal standard (*GAPDH*) and for total VEGF, as indicated. +, positive control sample. NAC reduced VEGF mRNA levels; one of 4 replicate experiments is shown.

similar to that *in vivo* (Fig. 3, *upper inset*). When NAC was added to the cultures after cell adhesion, 6 h after plating, and kept for an additional 18 h, the inhibition was less striking but still evident and statistically significant. The LI for VEGF in NAC-free controls was 21.0 ± 2.6, whereas in the presence of 0.1, 1, and 10 mM NAC, the LIs were 20.2 ± 4.1, 13.7 ± 2.1 ($P < 0.05$), and 5.3 ± 1.5 ($P < 0.001$), respectively.

The LI of untreated KS-Imm cells was very high for both Ki-67 (86.7%) and PCNA (97.9%), indicating that 24 h after plating almost all cells were in active proliferation. The addition of NAC did not affect these proliferation markers when added to the culture medium either at the moment of plating (Table 2) or 6 h later (data not shown).

Using a sensitive ELISA system, we observed that KS-Imm cells released a mean (± SD) of 236 ± 49 pg of VEGF/10^6 cells into the culture medium after a 24-h incubation. NAC-treatment of KS-Imm cells for 24 h strongly decreased the level of VEGF release into the medium in a dose-dependent manner; VEGF levels were reduced by 30% (to 70% of control) by 0.1 mM ($P < 0.01$) and by 45% (to 55% of control) by 1 mM NAC ($P < 0.001$). The addition of 10 mM NAC reduced the VEGF levels to below background ($P < 0.001$). Vitamin C also has strong antioxidant properties; treatment of KS-Imm cells with 0.1 mM vitamin C reduced VEGF levels by 20%, whereas 1 mM vitamin C reduced VEGF levels by 80% ($P < 0.001$).

A multiplex RT-PCR kit for analysis of the expression of several gene products involved in angiogenesis was used to determine the effects of NAC treatment on VEGF. Treatment with 10 mM NAC gave a substantial and consistent (67 ± 19% of that of controls) reduction of mRNA expression for total VEGFs (Fig. 5), whereas treatment with 1 mM NAC resulted in lower and more variable reduction in total VEGF mRNA levels. In the presence of vitamin C at 0.1 and 1 mM, VEGF mRNA levels strongly decreased (70% less; data not shown).

DISCUSSION

The antioxidant and antigenotoxic effects of the thiol NAC as well as its cancer preventive activity are well established (23, 25, 31). We demonstrated previously that NAC prevented tumor progression by modulating malignant tumor cell invasion *in vitro* and *in vivo*, either alone (25) or in synergism with doxorubicin (32, 33), apparently by inhibition of MMPs. Previous studies have shown that the unpaired cysteine in the propeptide of metalloproteases appears to chelate the zinc ion in the active site of the enzyme and block activity (34) and suggest that NAC inhibition of MMPs could be attributable to interactions between the thiol NAC and the zinc ion of the enzyme. NAC may also regulate collagenase production at the transcriptional level, because it has been reported that NAC inhibits the activation (35) and binding activity (36) of the transcription factor AP-1, which is involved in collagenase gene transcription (1). NAC has also been found to modulate a variety of gene expression and signal transduction pathways (35, 37–47).

The ability of NAC to inhibit angiogenesis (26) could readily explain the reduction of tumor take observed in earlier studies (25, 32, 33). Here we show that oral administration of NAC significantly inhibited the growth of established KS in both male and female mice and prolonged survival. The cause of death of the NAC-treated mice showing tumor regression is not clear. No overt pathologies were observed on autopsy, metastases were not observed, and no clear toxicity of NAC was apparent. It is possible this is related to persistent cachexia (see Ref. 48), although severe weight loss was not noted. Inhibition of MMP activity by NAC (25, 26) may be partially responsible for the KS inhibition effects; in fact, NAC inhibited KS cell gelatinase activity *in vitro* without altering the mRNA levels for these proteins. However, NAC also inhibited production of VEGF, a key angiogenic factor released in response to hypoxia and a major tumor angiogenic factor, the inhibition of which by blocking either the factor or its receptors has been shown to reduce tumor growth in preclinical models (reviewed in Ref. 5). NAC has been shown recently to inhibit VEGF production in cultured neuronal cells (49) and human melanoma cells (50), in agreement with the reduction of VEGF production by the highly angiogenic KS-Imm cells observed here. ROS appear to induce VEGF production (51); specific ROS generation may be a key part of the cellular oxygen tension detection system that regulates HIF-1a stabilization, and these ROS pathways are sensitive to thiols that maintain cellular GSH stores (52). NAC has been found to suppress the expression of VEGF induced by H_2O_2 in cultured rat heart endothelial cells (53). Rak *et al.* (54) demonstrated that NAC potently inhibits VEGF protein release from ras-transformed cells, whereas the effects of NAC on VEGF mRNA levels were limited. ROS species are also involved in several cellular signaling cascades, including ras (44, 47), which can also be inhibited by NAC. The observation that NAC inhibited VEGF protein secretion in cells engineered to overexpress an exogenous VEGF mRNA from the cytomegalovirus promoter further suggests that NAC affects VEGF production at posttranslational levels (54). Similarly, here we found that NAC reduced total VEGF mRNA levels by 50% at the highest concentration used, whereas it potently blocked VEGF protein production even at lower concentrations, suggesting both pre- and post-translational effects in KS-Imm cells.

When cells were treated with another known antioxidant, vitamin C, for 24 h at 0.1 and 1 mM, similar to NAC, vitamin C decreased VEGF mRNA and protein levels, with no effect on MMP-2 mRNA expression. Unlike NAC, vitamin C had no effect on MMP-2 activity in zymographic analysis, even at high concentrations. These data are consistent with antioxidant properties playing an important role in VEGF regulation, whereas the free thiol group of NAC, not present in vitamin C, appears to be critical for MMP-2 inhibition, as proposed previously (25, 26). Interestingly, unlike other antiangiogenic agents, NAC appears to inhibit angiogenesis in the absence of endothelial apoptosis, actually protecting these cells from apoptosis (55), further underscoring the safety of NAC as an anticancer agent. The NAC dose used *in vivo* (2 g/kg body weight) is the same that we used in previous experiments with other mouse strains, *e.g.*, C57BL/6 mice, for up to 180 days without any obvious side effects (25, 33). In humans, at a

well-tolerated dose of 1600 mg/m^2 body surface/day, corresponding to a daily dose of ~2700 mg, NAC plasma levels of 10 μg/ml were measured (56). These levels are very close (0.06 mM) to the 0.1 mM NAC used *in vitro*, which significantly decreased VEGF release by KS-Imm cells.

KS is particularly frequent in HIV-infected patients, where the use of HAART has led to decreased KS tumor burdens in AIDS-KS patients (57, 58). However, these patients are at very high risk for recurrence of KS once HAART is discontinued because of toxicity or other reasons (59, 60). Furthermore, KS is becoming a leading cause of cancer death in areas of Africa that have high rates of endemic KS (KSHV/HHV8 infection) along with HIV-1 infection (9). HAART is currently not an option for these patients largely because of economic factors. Therefore, identification of an inexpensive, nontoxic treatment for KS is urgently needed. The antiangiogenic activity of NAC on KS may partially fulfill this need. In addition, NAC (61) and thiols that are increased by NAC (62) have been shown to inhibit the replication of HIV-1, a major cofactor for KS.

The reasons for the clear sexual bias of KS is not known, although female hormones, and in particular pregnancy hormones, have been reported to curtail growth of KS tumor cell lines *in vivo* (16, 63). KS-Imm cells were derived from a male iatrogenic, posttransplant KS (16) and is one of the few "immortal" KS lines isolated to date, because cells from most primary cultures undergo senescence in culture. Here we observed a greater volume and more rapid growth profiles of KS-Imm tumors in untreated male mice as compared with female mice, in keeping with the preferential insurgence of KS in men. No overt differences in histology of the tumors between male and female mice were noted, however; thus, the mechanism of these apparent hormonal effects remains unclear.

In vivo, treatment of KS cells with NAC resulted in a significant reduction of proliferation indices and in foci of VEGF expression. There was a close correlation between these end points, suggesting that they were interrelated. It may be that NAC reduced VEGF expression, which in turn limited angiogenic activity that resulted in reduced proliferative potential in the tumors because of a shortage of nutrients. Alternatively, NAC could have inhibited cell proliferation, leading to relatively less development of hypoxia within the tumor, resulting in a lower induction of VEGF. Although either scenario would be compatible with the reduced tumor size observed in NAC-treated animals, our *in vitro* data suggest that NAC reduction of VEGF leads to inhibition of tumor growth. The method for determining the LI should be independent from the tumor size, in that a constant number of cells was calculated for each determination of PCNA or Ki-67, and a similar method was used to estimate expression of VEGF. *In vitro*, NAC significantly reduced VEGF expression, as determined by immunocytochemistry, ELISA, and multiplex RT-PCR, but did not effect proliferation indices. These data suggest that *in vivo* NAC treatment inhibited production of VEGF, thereby resulting in a reduced vascularization that in turn decreased the proliferative potential within these tumors.

The extensive clinical use of NAC for almost 40 years, mainly in the treatment of respiratory conditions, has established the safety and lack of toxicity of this drug even at high doses and for long-lasting treatments in humans. NAC has been shown to be an effective tumor chemopreventive agent; the observations that NAC inhibits MMP activity, angiogenesis, production of VEGF, and growth of highly vascularized KS indicate that prevention of tumor angiogenesis may play a key role. Our data suggest that effective tumor preventive agents may target tumor vascularization, a novel application for chemopreventive agents such as NAC. Accordingly, clinical studies evaluating the use of chronic NAC treatment in cancer patients as an antiangiogenic adjuvant therapy would be desirable.

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Inhibition of Angiogenesis-driven Kaposi's Sarcoma Tumor Growth in Nude Mice by Oral *N*-Acetylcysteine

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