Sulfasalazine-Induced Cystine Starvation: Potential Use for Prostate Cancer Therapy

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BACKGROUND. Certain cancers depend for growth on uptake of cystine/cysteine from their environment. Here we examined advanced human prostate cancer cell lines, DU-145 and PC-3, for dependence on extracellular cystine and sensitivity to sulfasalazine (SASP), a potent inhibitor of the x_c^- cystine transporter.

METHODS. Cultures were evaluated for growth dependence on exogenous cystine, x_c^- transporter expression, response to SASP (growth and glutathione content). In vivo, effect of SASP was determined on subrenal capsule xenograft growth.

RESULTS. Cystine omission from culture medium arrested DU-145 and PC-3 cell proliferation; both cell lines expressed the x_c^- transporter and were growth inhibited by SASP (IC_{50}s: 0.20 and 0.28 mM, respectively). SASP-induced growth inhibition was associated with vast reductions in cellular glutathione content—both effects based on cystine starvation. SASP (i.p.) markedly inhibited growth of DU-145 and PC-3 xenografts without major toxicity to hosts.

CONCLUSIONS. SASP-induced cystine/cysteine starvation leading to glutathione depletion may be useful for therapy of prostate cancers dependent on extracellular cystine. *Prostate 67: 162−171, 2007.* © 2006 Wiley-Liss, Inc.

KEY WORDS: x_c^-; cystine/glutamate antiporter; cystine starvation; glutathione; subrenal capsule xenograft

INTRODUCTION

Prostate cancer is the most common cancer as well as the second leading cause of cancer-related deaths for North American males. Most deaths from this disease are due to metastases that are resistant to conventional therapies. Although androgen ablation, currently the treatment of choice, can initially lead to substantial remissions, tumors frequently return in an androgen-independent form that is highly resistant to further hormonal therapy and other available regimens,

including chemotherapy. It is therefore of critical importance to identify new therapeutic targets and drugs for treatment of prostate cancer as it progresses in biological aggressiveness [1].

We have previously suggested a new approach for therapy of a variety of cancers [2,3]. It is based on inhibition of uptake of extracellular cystine (or the reduced form, cysteine) by cancers that cannot synthesize the amino acid and depend for growth on its availability from their micro-environment. Such malignancies include certain lymphomas [4], leukemias [5], and breast cancers [6]. While cysteine is required for protein synthesis in general, it is of critical importance for maintaining elevated levels of glutathione (GSH), a tripeptide thiol, consisting of glutamate, cysteine, and glycine, with a regulatory role in cell replication [7,8]. GSH has a short half-life and its biosynthesis is rate limited by the amount of available cysteine. A reduction in uptake of extracellular cystine/cysteine by cancer cells with a growth requirement for the amino acid can readily lead to depletion of their intracellular GSH content and subsequent growth arrest [8,9]. Evidence from various sources suggests that—in vivo—such cells can acquire cystine/cysteine mainly via two routes. Thus cystine, the predominant form of the amino acid in the circulation, can be taken up via the x_c^- cystine/glutamate antiporter, a plasma membrane cystine transporter that some cancer cells can express in particular when they are far advanced [4,10]. Alternatively, or in addition, the cells can readily take up extracellular cysteine as produced, for example, via an x_c^--mediated process by neighboring activated macrophages, dendritic cells, and fibroblasts; such somatic cells take up cystine via the x_c^- transporter, reduce it to cysteine and secrete cysteine into their micro-environment [3,11,12]. Based on such considerations one of us (P.W.G.) [13] initiated the idea that the x_c^- cystine/glutamate antiporter could provide a novel therapeutic target: its inhibition could lead to cystine/cysteine starvation and subsequent growth arrest of cancers dependent on supply of the amino acid from their micro-environment [4]. In a search for a therapeutically useful inhibitor, we found that sulfasalazine (salicylazosulfapyridine; SASP), an anti-inflammatory drug used for treatment of inflammatory bowel disease and rheumatoid arthritis, is a potent inhibitor of x_c^--mediated cystine uptake [2]. In vitro, SASP, used at levels reported in patients' sera (0.1–0.3 mM) [14], markedly inhibited growth of lymphoma and mammary cancer cell lines via specific inhibition of cystine uptake [2,6] and also inhibited cysteine secretion by fibroblasts without interfering with their proliferation [3]. Notably, intraperitoneal (i.p.) administration of SASP to rats led to marked growth arrest of lymphoma allografts without major toxicity to the rat hosts [2,3].

Taken together, the above observations suggest that SASP-induced inhibition of the x_c^- cystine/glutamate antiporter could form a viable strategy for therapy of a variety of cancers.

In the present study we investigated whether SASP could be useful as a drug for treating prostate cancer, in particular the androgen-independent form of the disease that has become resistant to conventional therapy. To examine the mechanism of action of SASP, we required a prostate cancer model permitting in vitro studies of cancer cell–drug interaction under highly controlled conditions. As well, the effect of SASP had to be determined on growth of tumors in experimental animals. In the absence of an ideal model of this type closely resembling the clinical situation [15], we used two well-established human prostate cancer cell lines, that is, DU-145 and PC-3, both in vitro and as subrenal capsule xenografts in immuno-deficient mice [15]. The two cell lines, representing advanced, androgen-independent prostate cancer [16], were examined for growth dependency on exogenous cystine and sensitivity to treatment with SASP. It was found that the proliferation of DU-145 and PC-3 cells critically depends on uptake of extracellular cystine. Moreover, their growth was highly sensitive to SASP both as cultures and as xenografts.

MATERIALS AND METHODS

Materials and Animals

Chemicals, stains, solvents, and solutions were obtained from Sigma-Aldrich Canada Ltd, Oakville, ON, Canada, unless otherwise indicated. Eight- to ten-week-old male NOD-SCID and Rag-2M mice were bred by the BC Cancer Research Centre Animal Resource Centre, BC Cancer Agency, Vancouver, Canada.

Cell Cultures

Human prostate cancer cell lines DU-145 and PC-3 were obtained from the American type culture collection (Manassas, VA). Cultures were maintained in RPMI-1640 medium (Stem Cell Technologies, Vancouver, BC, Canada), supplemented with heat-inactivated fetal bovine serum (FBS; 10%; Gibco-BRL, Burlington, ON, Canada), penicillin (50 U/ml), and streptomycin (50 µg/ml) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C; for harvesting of cells 0.25% trypsin-1.0 mM EDTA solution (Gibco-BRL) was used. For experiments, cells were used from a stock supply of cell aliquots frozen in liquid nitrogen; cultures were monitored for changes in growth characteristics and doubling times and maintained for not more than 20 passages.

Assessment of Growth Requirements for Exogenous Cystine

Following trypsinization of near-confluent monolayer cultures, cells were centrifuged (4 min at $100g$) and resuspended in fresh maintenance medium. Aliquots (1.0 ml; 12,000 cells/ml) were distributed in 12-well plates (Linbro, Flow Laboratories, Mississauga, ON, Canada) and incubated overnight to allow cell attachment. After cells had adhered, the medium was removed without disturbing the monolayers and following a rinse with PBS (pH 7.2, 37°C), fresh cystine- and methionine-deficient RPMI-1640 medium, supplemented with dialyzed FBS (10%) and antibiotics, was added (0.8 ml). Cystine (0.2 mM), methionine (0.1 mM), cystathionine (0.15 mM), or cystine/methionine-deficient medium was added in various combinations (final volume 1.0 ml; triplicates) for a 5-day incubation, with a medium change after 3 days. On day 5, cells were harvested and their numbers determined using an electronic cell counter (Beckman-Coulter, Hialeah, FL). Results are presented as percentage growth (mean $\pm$ SD) relative to controls (cultures in medium containing cystine and methionine).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Log phase cultures of DU-145 and PC-3 cells in maintenance medium were trypsinized, centrifuged, and the cell pellets were snap frozen in liquid nitrogen for subsequent assay of xCT mRNA expression. To this end, total RNA was isolated using RNAzol-B (Tel-test, Friendswood, TX) and quantified spectrophotometrically [2,6]. cDNAs from 10 μg RNA were generated using AMV reverse transcriptase (Promega, Madison, WI). Oligonucleotide primers and probes (6FAM, reporter dye) for the human xCT subunit of x_c^- (GenBank accession no. AB026891) and β-actin were designed for use in TaqMan real-time PCR assay (PE Applied Biosystems, Foster City, CA). A reaction mixture was prepared containing primers and probes at optimized final concentrations to which template cDNA was added in triplicate wells of optically clear 96-well reaction plates. An ABI PRISM 7000 Sequence Detection System was used to measure fluorescence during the PCR procedure [50°C for 2 min and 95°C for 10 min, followed by 15 sec at 95°C (melting step), and 1 min at 60°C (anneal/extension) for 40 cycles]. Data were analyzed using a relative quantitation technique employing sequence detection systems software. Each sample (triplicates) was normalized to levels of β-actin mRNA within each sample. The mean level of expression from each sample was then compared to an internal reference.

SASP Solutions

For in vitro studies a 10 mM SASP solution was prepared, under subdued light conditions, by dissolving 40.1 mg of the drug (using a magnetic stir bar) in 4.0 ml 0.1 N NaOH plus 5.77 ml PBS (pH 7.2). The pH of the solution was then adjusted to approximately 7.5 by slow addition of 5×50 μl 1.0 N HCl and the solution was filter-sterilized (0.2 μm syringe filter, Millipore, Carrigtwohill Co., Cork, Ireland). Appropriate dilutions were prepared for immediate addition to cell cultures, using Fischer's medium or minimum essential medium (MEM), supplemented with heat-inactivated FBS (10%) and antibiotics. For in vivo studies, SASP solutions were prepared at 20 mg/ml, for example, 400 mg SASP in 15.0 ml 0.1 N NaOH plus 4.80 ml PBS (pH 7.2); the pH was lowered to about 8 by addition of 2×100 μl 1.0 N HCl and the solution filter-sterilized [2].

Testing of SASP on Culture Growth and Total GSH Levels

Following trypsinization of near-confluent DU-145 and PC-3 cell cultures, cells were centrifuged (4 min at $100g$) and resuspended in maintenance medium to concentrations ranging from 12,000 to 25,000 cells/ml. Aliquots (2.0 ml) were distributed in 12-well culture plates (Linbro) and incubated overnight to allow cell attachment. After adherence of the cells, the medium was removed, and following a rinse with PBS (37°C), fresh Fischer's medium, supplemented with FBS (10%) and antibiotics, was added (1.8 ml). To determine the effect of SASP on cell proliferation, cultures (in triplicate) received additions of SASP (at a range of concentrations), 2-mercaptoethanol (2-ME; 66 μM) or Fischer's medium in various combinations (final volume 2.0 ml) for a 72-hr incubation. At the end of this period the medium was removed without disturbing the monolayers, cells were trypsinized and cell numbers determined using an electronic cell counter. Culture growth inhibitions were calculated from the increases in cell numbers over the 72-hr period in SASP-treated cultures and their controls using the formula: Percent inhibition $= 100 - ($increase$_{\text{SASP-treated}}/$increase$_{\text{control}}) \times 100$. Data are presented as mean $\pm$ SD. Fischer's medium was used instead of RPMI-1640 since it contains cystine at about 84 μM, approximating physiological levels in the peripheral blood [17]. In contrast, RPMI-1640 contains much higher cystine levels (207 μM) shown to reduce the action of SASP [2].

To determine the effect of SASP on intracellular GSH levels, cultures were incubated with SASP in the absence and presence of 66 μM 2-ME (as above) but, instead of Fischer's medium, MEM was used containing about the same concentration of cystine. Total GSH

levels were determined using an ApoGSH^{TM} Glutathione Colorimetric Detection kit from BioVision Research Products (Mountain View, CA) and following the manufacturer's instructions. Cell viability was determined using the Neutral Red cytotoxicity test. Data are presented as amounts of GSH in ng/10^6 viable cells.

Testing of SASP In Vivo

DU-145 or PC-3 cells were suspended in unpolymerized rat-tail collagen, which was allowed to polymerize. Using a grafting procedure described in detail elsewhere [15,18], the resultant collagen gels were grafted (2×10^6 cells/gel) under renal capsules (one graft per kidney) of NOD-SCID mice supplemented with testosterone via subcutaneously implanted testosterone pellets (10 mg/mouse), as previously described [19]. Harvested tumors (volume approximately 200 mm^3) were cut into $3 \times 3 \times 1$ mm pieces for re-grafting under the kidney capsules of groups of testosterone supplemented, male Rag-2M mice (two grafts per kidney). In addition, sections of the tumors were collected for histological analysis. When the tumor transplants reached an average volume of about 20–50 mm^3 (as determined in replicate mice), the animals were randomly sorted into control and treatment groups (six mice/group) and received, under subdued light conditions, i.p. injections (every 12 hr) of saline (controls) or SASP (250 mg/kg body weight) for periods up to 14 days. At the start and finish of the treatment with SASP, animals were sacrificed for necropsy and tumors harvested, measured, photographed, and fixed for histopathological analysis. Tumor size was expressed in mm^3, using the formula: volume (mm^3) $= 0.52 \times$ length $\times$ width $\times$ height (in mm). Data are presented as mean $\pm$ SEM. Fresh SASP solutions were prepared every day. Food and water were provided to the mice ad libitum; their health was monitored daily for stress-related effects, including excessive weight loss and abnormal behavior. Animal care and experiments were carried out in accordance with the guidelines of the Canadian Council on Animal Care.

Immunohistochemistry

Pre- and post-graft tumor tissues were fixed in 10% neutral buffered formalin, processed through alcohols, and embedded in paraffin. Sections were cut by microtome at a thickness of 4 µm and mounted on glass slides. For immunohistochemical staining, sections were dewaxed in Histoclear (National Diagnostic, Atlanta, GA) and hydrated in graded alcohol solutions and distilled water. Staining was carried out using hematoxylin and eosin (H&E); mouse anti-human Ki-67 antibody (dilution 1:50; DAKO, Mississauga, ON, Canada), lacking cross reactivity for mouse Ki-67, was used as a proliferation marker and an Apoptag Apoptosis Detection kit (Serological Corporation, Norcross, GA) was used to assess the degree of apoptotic cell death, as previously described [20].

Statistical Analysis

Tumor volumes and proliferation rates (i.e., Ki-67 labeling) in SASP-treated and control groups of mice were compared for statistically significant differences using ANOVA and the Student's t-test.

RESULTS

Growth Requirements for Exogenous Cystine In Vitro

Cysteine can be generated in tissues (e.g., liver) by methionine metabolism via the transsulfuration pathway involving cleavage of cystathionine by γ-cystathionase to α-ketobutyrate and cysteine [21]. In contrast, certain types of cancer cells are not able to generate cysteine and, to sustain growth, require uptake of the amino acid from their micro-environment [2,5,6]. To determine if DU-145 and PC-3 prostate cancer cells could proliferate in the absence of exogenous cystine, the two cell lines were cultured in medium from which cystine had been omitted; the effect of omission of methionine, a nutritionally essential amino acid, was also studied as a positive control. In addition, it was determined whether cystathionine could act as a substitute for cystine. It was found that whereas the DU-145 and PC-3 cell lines grew actively in complete culture medium (containing 10% dialyzed FBS), specific omission of cystine or methionine from this medium led to abrogation of culture growth in both cases (Fig. 1). Furthermore, cystathionine, which has been reported to fully replace cystine for certain cell cultures [22], could not act as a cystine substitute when added to cystine-deficient, methionine-containing DU-145, or PC-3 cultures; it also had no effect on the growth of control cultures. Taken together, the results indicate that DU-145 and PC-3 cells are critically dependent for growth on uptake of cystine/cysteine from their micro-environment and lack an operative methionine-cystathionine-cysteine pathway.

Expression of the x_c^- Cystine Transporter

The x_c^- cystine transporter is a heterodimeric plasma membrane protein consisting of a heavy subunit, 4F2hc, and a light subunit, xCT, linked by a disulfide bridge. The 4F2hc subunit is a common component of amino acid transporters with a role in
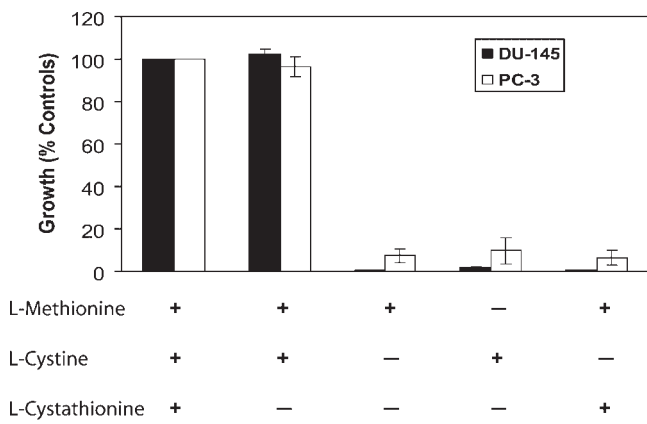
Fig. I. Effect on growth of human DU-145 and PC-3 prostate cancer cell cultures of omission of cystine or methionine from the culture medium, substitution of cystine by cystathionine. After an overnight incubation in RPMI-1640 medium supplemented with dialyzed FBS (10%) and antibiotics, monolayer cultures were rinsed with PBS and further incubated for 5 days with culture medium lacking or containing cystine (0.2 mM), methionine (0.1 mM), or cystathionine (0.15 mM), in the combinations indicated and as described in Materials and Methods. Cell numbers were determined using an electronic cell counter. The data are representative of results from three experiments and are expressed as percentage growth relative to controls (mean ± SD).

Fig. 2. Expression of xCT mRNA in DU-145 and PC-3 cells from exponentially growing cultures as shown by RT-PCR. The xCT subunit levels were normalized to levels of β-actin mRNA within a sample (triplicates) and the mean levels of expression normalized to that of DU-145 (see Materials and Methods). Data are presented as mean ± SEM and representative of results from two experiments.

anchoring the heterodimer to the plasma membrane; the xCT subunit controls cystine transport function and specificity [23]. Since the 4F2hc subunit is generally present in cells, we only determined the expression in the prostate cancer cells of the cystine-specific xCT subunit. As shown in Figure 2, both the DU-145 and, in particular, the PC-3 cell line showed expression of the *xCT* gene as measured via RT-PCR, suggesting expression by both cell lines of the x_c^- cystine transporter. This is consistent with the ability of both cell lines to readily proliferate in 10% FBS-supplemented Fischer's medium containing relatively low cystine levels approximating those in the peripheral blood (average culture doubling times: 39 and 31 hr for DU-145 and PC-3 cultures, respectively). The higher *xCT* expression by the PC-3 cells suggests that they have more x_c^- cystine transporters per cell than the DU-145 cells and hence a greater ability to take up cystine from the microenvironment.

Effect of SASP on Culture Growth

SASP inhibited growth of DU-145 and PC-3 cultures in Fischer's medium supplemented with dialyzed FBS (10%) with IC_{50}s (±SD) of 0.20 (±0.01) and 0.28 (±0.02) mM, respectively (Fig. 3A). Essentially complete growth inhibition of DU-145 and PC-3 cultures was obtained at SASP concentrations of 0.3 and 0.4 mM, respectively. As shown in Figure 3B, growth inhibition
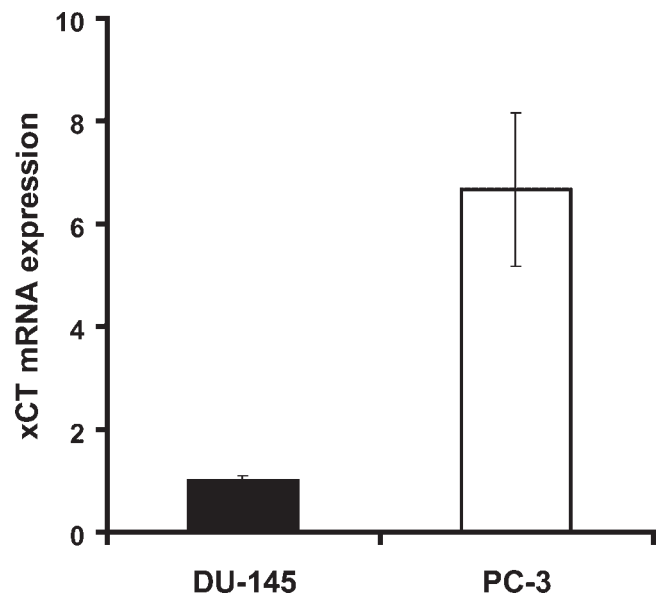
could almost completely be prevented by addition of 2-ME (66 µM), which at such low concentrations allows cellular uptake of cystine (as a mixed disulfide of 2-ME and cysteine) via the leucine transporter [24], thus bypassing the x_c^- cystine transporter. This finding indicates that the growth arrest of DU-145 and PC-3 cultures by SASP at concentrations = 0.3 and 0.4 mM, respectively, was the result of highly specific inhibition of x_c^--mediated cystine uptake, as previously observed with lymphoma cell cultures [2]. The lower sensitivity of the PC-3 cells to SASP is consistent with a higher number of x_c^- cystine transporters per cell as suggested by their higher *xCT* expression (Fig. 2).
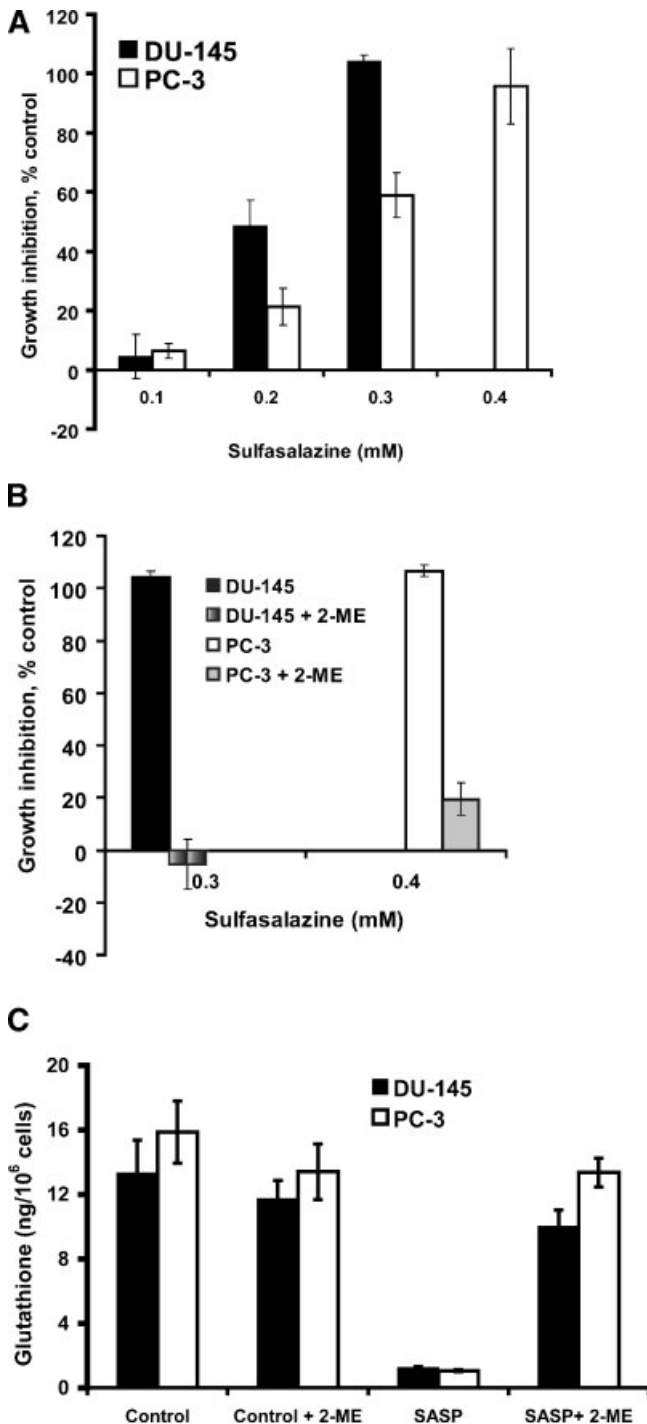
Effect of SASP on Intracellular GSH Levels

As shown in Figure 3C, a 24-hr incubation of DU-145 and PC-3 cells with 0.3 and 0.4 mM SASP, respectively, led to marked depletion of their intracellular total GSH contents. Thus GSH levels decreased to 9.3% (SD = 0.01) and 6.6% (SD = 0.01) of normal values for DU-145 and PC-3 cells, respectively, that is, a reduction >90%. The cells treated with SASP for 24 hr in the absence of 2-ME were viable as indicated by the Neutral Red cytotoxicity assay, showing 95% and 85% viability for DU-145 and PC-3 cells, respectively. The SASP-induced reduction in GSH levels was completely prevented by 2-ME (66 µM), indicating that it stemmed

from inhibition of cystine uptake via the x_c^- cystine transporter.

Effect of SASP on Xenograft Growth

In view of the above results, we initiated preliminary studies of the effect of SASP on xenografts of the two cell lines. Mice carrying actively growing DU-145 and PC-3 subrenal capsule xenografts were treated with SASP (250 mg/kg body weight; i.p.; b.i.d.) for 14 and 7 days, respectively. The renal graft site was used since it offers very high tissue perfusion and potentially rapid development of graft microvasculature [15]. SASP was administered intraperitoneally to avoid intra-intestinal cleavage to sulfapyridine and 5-amino-salicylic acid [27], and ensuing loss of x_c^--inhibitory activity [2]. SASP markedly inhibited the increase in tumor size of both cell lines, with growth inhibitions of 35%–56% for DU-145 tumors (Fig. 4A) and up to 80% for PC-3 tumors (Fig. 4B). The treatments with SASP did not lead to major toxic side-effects in the hosts. Tumor growth, however, recurred following discontinuation of the treatment with SASP (data not shown).

Histological Analysis of Tumor Tissues

H&E-stained sections of control PC-3 xenografts showed tumor cells arranged in solid nests forming abortive glandular lumina, a well-developed microvasculature and minimal apoptosis or necrosis (Fig. 5A); DU-145 tumors showed a similar histology (data not shown). Examination of PC-3 tumor sections showed that the SASP-treated tumors were very similar to the control tumors with regard to histology and extent of tumor necrosis (Fig. 5A,B); as well, there were no significant differences in apoptosis between control and SASP-treated tumors (data not shown). The Ki-67 proliferation index of the SASP-treated tumors, however, was significantly lower, that is by 28.4% ($P = 0.01$) (Fig. 5C,D). This is consistent with the lower rate in tumor volume increase observed in SASP-treated animals (Fig. 4). The results indicate that SASP inhibited the growth of the tumors by cytostatic rather than cytolytic action.

DISCUSSION

In the present study we have shown that human DU-145 and PC-3 prostate cancer cells are critically

Fig. 3. Effect of SASP on growth and GSH content of DU-145 and PC-3 cell cultures: prevention by 2-ME. **A**: Cultures of the cells in Fischer's medium, supplemented with FBS (10%) and antibiotics, were incubated for 72 hr with SASP at the concentrations indicated and in the absence of SASP (control). **B**: In parallel cultures, incubation was carried out with added 2-ME (66 μM) to allow cellular uptake of cystine via a route circumventing the x_c^- transporter (see text). In both cases, cell numbers were determined using an electronic cell counter. Data are presented as "growth inhibition relative to controls (no SASP)" (mean ± SD) and are representative of results from three experiments. **C**: DU-145 and PC-3 cells in MEM, supplemented with FBS (10%) and antibiotics, were incubated for 24 hr with 0.3 and 0.4 mM SASP, respectively, both in the absence and presence of 2-ME (66 μM), and their GSH contents and viability determined (see Materials and Methods). GSH levels/10^6 viable cells are expressed as mean ± SD; results are representative of two experiments.

dependent on extracellular cystine for growth. This was shown by their lack of growth in cystine-deficient medium (Fig. 1). The requirement of these cells for exogenous cystine is also illustrated by SASP-induced growth arrest in vitro that was specifically due to

cystine starvation. Thus this growth arrest, as well as SASP-induced GSH depletion, could essentially completely be prevented by 2-ME (66 µM), which allows cellular cystine uptake via a route circumventing the x_c^- transporter [24] (Fig. 3A–C). [It may be noted that 2-ME at a concentration of ~66 µM does not affect the molecular structure of SASP, as shown by us using high performance liquid chromatography of SASP samples treated with 2-ME for extended periods (unpublished observations).] In addition, the failure of cystathionine to substitute for cystine (Fig. 1) indicates that the two cell lines are not able to synthesize cysteine. The above results therefore suggest that cystine/cysteine starvation leading to GSH depletion could be useful for therapy of prostate cancers that—similar to the DU-145 and PC-3 cell lines—depend for growth on uptake of the amino acid from their micro-environment. A similar suggestion has been made for other malignancies, including certain human lymphomas and leukemias [2,5,22], breast cancers [6], and, very recently, gliomas [25]. On the other hand, primary prostate cancers might, in contrast to cultured DU-145 and PC-3 cells, be able to synthesize cysteine and hence be resistant to cystine/cysteine starvation. Such resistant cancers would express γ-cystathionase, the enzyme involved in the last step of cysteine biosynthesis [21], and could be identified via immunohistochemical assessment of the enzyme in biopsy specimens [26].

Expression of the x_c^- cystine transporter by DU-145 and PC-3 cells, as indicated by gene expression of the xCT subunit (Fig. 2), is consistent with their requirement for exogenous cystine, the predominant form of the amino acid in the circulation or culture medium. In fact, the x_c^- cystine transporter appears to be essential for growth of the two cell lines in vitro, since complete growth inhibition could be obtained via specific inhibition of its function by SASP (Fig. 3A,B), a potent inhibitor of x_c^--mediated cellular cystine uptake as first described by us [2]. The lower sensitivity of PC-3 cells to SASP in vitro, relative to DU-145 cells (Fig. 3), can be explained by a higher expression of the x_c^- transporter
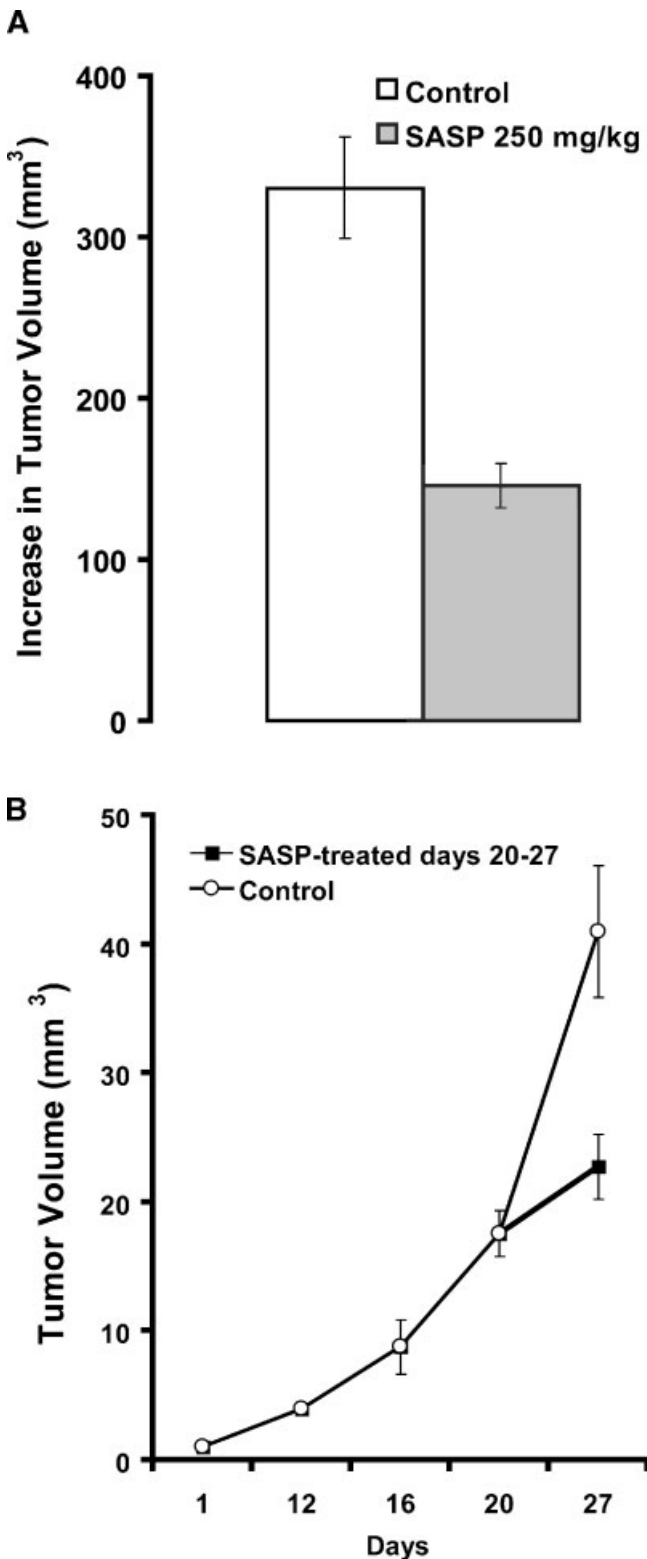
Fig. 4. Effect of SASP on growth of human prostate cancer cell xenografts in Rag-2M mice. Groups of mice (n = 6) carrying subrenal capsule tumors of DU-145 or PC-3 cells were given i.p. injections of saline (control) or SASP (250 mg/kg body weight) twice a day for one or two weeks starting when tumors became well-established (average tumor volume in the range 20 – 50 mm³). The animals were then sacrificed for determination of tumor volumes and immunohistochemical analysis (see Materials and Methods). Data are expressed as "increase in tumor volume" or "tumor volume" (mean ± SEM) and are representative of results from at least two experiments. **A**: SASP treatment of DU-145 tumors for 2 weeks resulting in tumor growth inhibition of 56% (P < 0.001); (**B**) SASP treatment of PC-3 tumors for 7 days, starting on day 20 resulting in tumor growth inhibition of 80% (P = 0.001).
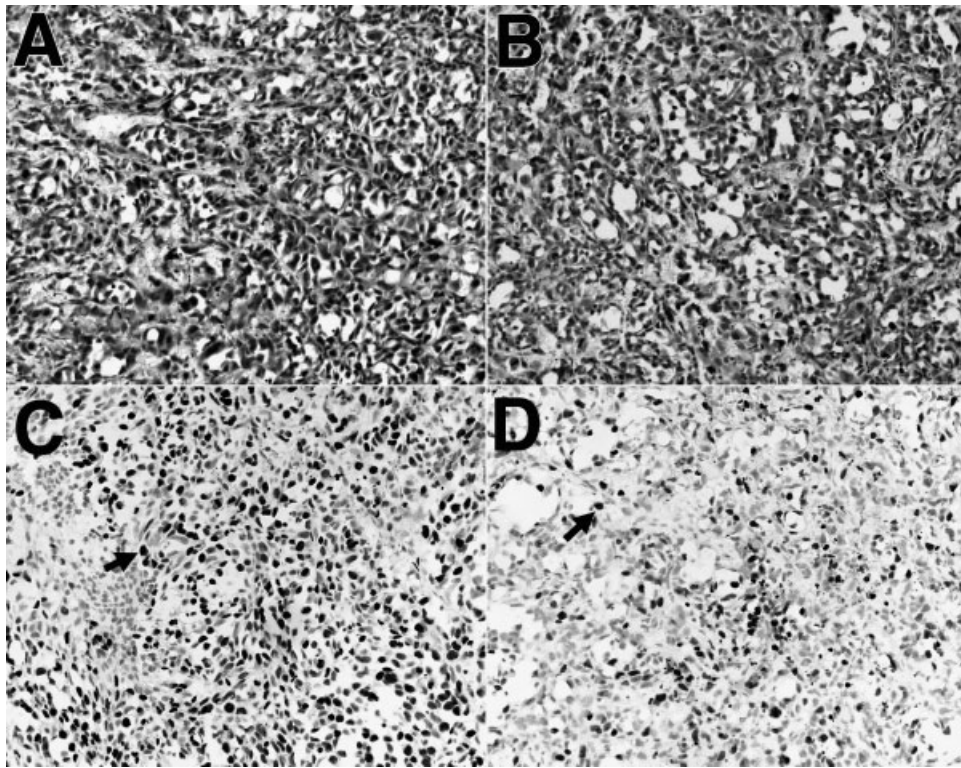
Fig. 5. Tissue sections from control (saline treated) and SASP-treated PC-3 tumor subrenal capsule xenografts (see Fig. 4B) subjected to immunohistochemistry as described in Materials and Methods, (original magnification, 200×). H&E-stained tissues: **A**, control and **B**, SASP-treated. Ki-67-labeled tissue: **C**, control, and **D**, SASP-treated. Arrows indicate Ki-67-labeled nuclei.

in the PC-3 cells, generating a requirement for higher concentrations of the drug to inhibit the transport of cystine.

SASP markedly inhibited the proliferation of DU-145 and PC-3 cells both in vitro and in immuno-deficient Rag-2M mice carrying subrenal capsule xenografts of the cells (Fig. 3A,B and Fig. 4A,B). The growth-inhibitory effect of SASP in vivo was indicated both by a marked reduction in the rate of tumor volume increase and a reduction in the Ki-67 proliferation index of the cancer cells, as shown with an antibody specific for human Ki-67 (Fig. 5C,D). It is not clear why the PC-3 tumors appear to be more sensitive to SASP than DU-145 tumors, since DU-145 cells in vitro exhibited greater sensitivity to the drug. The absence of increased cell death in the SASP-treated tumors (Fig. 5B) indicates that SASP inhibited growth of the prostate tumor transplants via cytostatic rather than cytolytic action. The lack of major side-effects of SASP in the hosts is consistent with the low toxicity associated with its use in the clinic [27], in our previous studies with rats [2,3] and in studies by others using mice [25].

The growth-inhibitory activity of SASP in DU-145 and PC-3 cultures is likely based on depletion of intracellular GSH resulting from cystine starvation (Fig. 3A–C). The GSH contents of the cells were already >90% depleted after 24-hr of incubation with SASP (Fig. 3C). GSH is well-known for its protection of cells from metabolism-generated free radicals [7] and its intracellular depletion has been demonstrated to lead to reduced neutralization of oxidative stress and subsequent growth arrest of cells [8]. In contrast, the basis of the anticancer activity of SASP in vivo (Fig. 4) is not as clear, although it probably involves reduction of GSH levels in the cancer cells. Support for this suggestion comes from a recent study by Chung et al. [25] who worked with human glioma cells whose GSH synthesis relied primarily on cystine uptake via the x_c^- transporter. They found that intraperitoneal administration of SASP to immuno-deficient mice carrying glioma xenografts led to growth inhibition of the transplants associated with a reduction in their GSH levels. Such a reduction in GSH content of tumor transplants would be consistent with cystine/cysteine starvation via SASP by targeting (i) x_c^--mediated cystine uptake by the cancer cells and (ii) x_c^--mediated supply of cysteine by tumor-associated immune cells, as previously suggested by us [2,3]. In the present study, SASP was administered to the animals twice daily in an effort to keep the tumors exposed to the drug

at continuously elevated levels. However, the actual concentration of SASP within the tumor micro-environment is not known. Whereas SASP inhibits x_c^--mediated cystine uptake at concentrations as low as ~0.1 mM [2], it can also inhibit, at concentrations >0.5 mM, activation of nuclear factor kappa B (NFκB), a transcription factor with a regulatory role in cell proliferation and apoptosis [28]. It can therefore not be excluded that the growth-inhibitory effects of SASP on the DU-145 and PC-3 xenografts may in part be due to anti-NFκB activity. Furthermore, treatment with SASP may reduce the number of tumor-associated macrophages (TAMs), that is, non-neoplastic somatic cells that have recently been recognized for a stimulatory role in tumor growth via secretion of, for example, cytokines [29]. Thus, SASP has been reported to interfere with cytokine production by macrophages and, when administered intraperitoneally to mice, can readily lead to apoptosis of peritoneal macrophages [30]. Initial studies on the effect of SASP on TAMs have shown that intraperitoneal administration of SASP (250 mg/kg, b.i.d.) led to a substantial reduction in the number of macrophages associated with subrenal capsule tumors of LNCaP prostate cancer cells in Rag-2M mice [31]. This significant finding warrants further study.

While GSH is best known as a cellular antioxidant, it also has a major role in resistance of cancers to chemotherapy, as it is involved in detoxification and drug extrusion from cells via multidrug-resistance proteins [10,32]. There is an increasing amount of evidence that depletion of GSH in target cells enhances therapeutic efficacy of anticancer agents and hence provides a new strategic approach urgently needed in cancer therapy [9,33,34]. Elevated GSH levels in malignant cells can apparently to a large extent be maintained via the x_c^- transporter which enables cells to quickly take up extracellular cystine for biosynthesis of GSH and increased drug resistance. In view of this, it appears that the x_c^- cystine transporter is not only useful as a target for inhibition of cancer growth via cystine/cysteine starvation therapy, but also for reducing drug resistance of cancers via GSH depletion [8,9,34]. In this context it is of major interest that SASP has been found to sensitize cancer cells to chemotherapeutic agents, both in vitro [9] and in experimental animals [35]. Since in the present study SASP on its own arrested the growth of the prostate cancer xenografts but did not induce apoptosis (Fig. 4 and Fig. 5), it could be more effective when used in combination with other drugs especially those producing free radicals. This suggestion will be addressed in follow-up studies.

SASP is administered orally when used for treatment of severe inflammatory diseases—a route not very suitable for its application as an anticancer agent, since it leads to its marked degradation by intestinal bacteria and ensuing loss of x_c^--inhibitory activity [2,27]. However, this degradation of SASP can be substantially reduced by using oral administration of SASP in combination with antibiotics [27,36].

CONCLUSIONS

The present study suggests that SASP, an FDA-approved, anti-inflammatory drug, may be useful for chemotherapy of advanced prostate cancers that have a growth requirement for extracellular cystine/cysteine as found for DU-145 and PC-3 prostate cancer cells. By inducing depletion of GSH levels in target cells, SASP could be particularly useful as a sensitizer for a second anticancer agent. The fact that SASP is a relatively non-toxic and inexpensive drug should facilitate its clinical use as a new anticancer agent.

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