In vitro studies on the lymphoma growth-inhibitory activity of sulfasalazine

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Sulfasalazine (SASP) is a novel, potent inhibitor of cellular cystine uptake mediated by the x_c^- cystine/glutamate antiporter. Lymphoid cells cannot synthesize cyst(e)ine and depend for growth on its uptake from their micro-environment. We previously showed that SASP (0.2 mM) can abrogate lymphoma cell proliferation *in vitro* by specifically inhibiting x_c^--mediated cystine uptake. Intraperitoneal administration of SASP to Noble rats markedly suppressed Nb2-U17 rat lymphoma transplant growth, notably without major toxicity to the hosts. Since Nb2-U17 cells are x_c^--deficient, the growth arrest was apparently not due to SASP–tumor cell interaction, but possibly to interference with x_c^--mediated cysteine secretion by somatic cells. In this study we found that replication of x_c^--deficient Nb2-11 lymphoma cells can be sustained *in vitro*, in the absence of cystine uptake enhancers, by co-culturing with IMR-90 fibroblasts known to secrete cysteine. SASP, at 0.15 and 0.2 mM, arrested replication of fibroblast-driven Nb2-11 cells by 93 and 100%, respectively, without impeding fibroblast proliferation. Addition of 2-mercaptoethanol (60 μM), a cystine uptake enhancer, almost completely prevented this growth arrest, indicating that SASP specifically inhibited cysteine secretion by the fibroblasts, a process based on x_c^--mediated cystine uptake. It is proposed that the lymphoma growth-inhibitory activity of SASP *in vivo* involves inhibition of cysteine secretion by tumor-associated somatic cells (macrophages, dendritic cells), leading to cysteine starvation of the tumor cells and apoptosis. The difference between the lymphoma cells and fibroblasts in sensitivity to SASP treatment is consistent with the marked antitumor effect of SASP lacking significant side effects. *Anti-Cancer Drugs* 14:21–29 © 2003 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2003, **14**:21–29

Keywords: anticancer drug, dendritic cell, lymphoma, macrophage, mechanism of action, sulfasalazine, x_c^- cystine/glutamate antiporter

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Sponsorship: Supported by the BC Cancer Foundation with core support (P.W.G.) from the BC Cancer Agency, Vancouver, Canada.

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Received 27 August 2002 **Accepted** 9 October 2002

Introduction

The amino acid, cystine, or its reduced form, cysteine, is essential for cell growth and metabolism. Cyst(e)ine deficiency within mammalian cells leads to a rapid decline in their content of glutathione, a tripeptide thiol produced from glutamate, cysteine and glycine, which is a major scavenger of free radicals [1] and also has a role in control of cell replication [2]. The decrease in glutathione levels in turn leads to loss of cellular defense against metabolism-generated oxidative stress, arrest of cell proliferation and, ultimately, apoptosis [3,4]. Lymphoid cells cannot synthesize cyst(e)ine, and depend for growth and viability on uptake of the amino acid from their micro-environment [5]. Cancers originating from lymphoid tissue, e.g. lymphomas and leukemic cells, have been found to retain the inability to synthesize cyst(e)ine; such malignancies are therefore potentially sensitive to therapy based on cyst(e)ine starvation [6,7].

Lymphoid cells typically exhibit a low uptake capability for cystine, as distinct from cysteine which they can take up readily [8]. Culture medium, however, predominantly contains cystine; cysteine provided in certain media is rapidly oxidized to cystine. Consequently, growth of lymphoid cell cultures in general requires the presence in the medium of a cystine uptake enhancer such as 2-mercaptoethanol (2-ME) or elevated levels of cystine [7,9]. Alternatively, the cultures can be maintained using feeder layers of fibroblasts or macrophages, which can continuously provide the lymphoid cells with cysteine [10]. The latter process is based on the ability of such somatic cells to take up extracellular cystine via a plasma membrane cystine/glutamate antiporter (x_c^-) [11], reduce the amino acid intracellularly and secrete it as cysteine which the lymphoid cells can take up readily. Apparently this is a major mechanism *in vivo* by which lymphocytes can be supplied with cysteine critically required for their proliferation [12,13].

We have previously suggested that the x_c^- cystine transporter provides a potentially useful target for therapy of lymphoid cancers. Its inhibition *in vivo* would presumably not only inhibit cystine uptake by lymphoma cells expressing an x_c^- cystine transporter, as found for

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certain lymphoma cells following malignant progression, but also interfere with the supply of cysteine to the target cells by somatic cells (e.g. macrophages, fibroblasts) [7]. It was envisioned that short-term, drug-induced inhibition of the x_c^- cystine transporter *in vivo* could promote elimination of lymphoma cells by cyst(e)ine starvation, hopefully without major side effects to the host [7].

Recently, we demonstrated that sulfasalazine (salicylazosulfapyridine, SASP), an immuno-suppressant used for decades in the therapy of Crohn's disease and rheumatoid arthritis [14], is a novel and potent inhibitor of the x_c^- cystine transporter, in contrast to its colonic metabolites, sulfapyridine and 5-aminosalicylic acid [15]. *In vitro*, SASP abrogated rat Nb2-SFJCD1 lymphoma cell replication, at patient-tolerated levels (0.2 mM), via specific inhibition of x_c^--mediated cystine uptake; SASP was even more effective against human non-Hodgkin's lymphoma (DoHH2) cultures. Intraperitoneal administration of SASP to rats led to very marked growth inhibition of well-developed, s.c. Nb2-U17 rat lymphoma transplants, notably without significant toxicity to the hosts. As such, SASP exhibits major potential for use in chemotherapy of lymphoid cancers [15]. Importantly, Nb2-U17 lymphoma cells do not express the x_c^- cystine transporter, have a low uptake capability for cystine and, *in vitro*, display only low sensitivity to SASP when supplied with adequate amounts of cysteine [15]. This suggests that the arrest of the Nb2-U17 lymphoma growth in rats by SASP was not the result of direct drug–tumor cell interaction, but possibly due to an interference by SASP with the secretion by somatic cells of cysteine required for the proliferation of the tumor cells. In the present study we have investigated this possibility using co-cultures of fibroblasts and x_c^--deficient Nb2 lymphoma cells in which the proliferation of the lymphoma cells, in the absence of a cystine uptake enhancer, critically depended on secretion by the fibroblasts.

Materials and methods

Materials

SASP, chemicals and culture medium were obtained from Sigma-Aldrich (Oakville, ON, Canada), unless otherwise indicated.

Cell cultures

Nb2-U17 and Nb2-11 rat pre-T lymphoma cell lines, developed in our laboratory [16], were maintained as suspension cultures in 80 cm2 tissue culture flasks (Nunc; VWR Canlab, Mississauga, ON, Canada) in Fischer's medium supplemented with fetal bovine serum (FBS, 10%; Gibco, Invitrogen Canada, Burlington, ON, Canada), penicillin (50 U/ml), streptomycin (50 μg/ml) and 2-ME (60 μM) at 37°C in a 5%CO_2/air atmosphere, as previously described [7,15,16]. Both cell lines are critically dependent for growth on lactogens, present in FBS, and on 2-ME for mediation of cystine uptake [7,16]. The Nb2-11 cell line is available from the European Collection of Cell Cultures (ECACC, Salisbury, UK). IMR-90 normal human lung fibroblasts (cat. no. CCL-186; ATCC, Manassas, VA) were similarly cultured, but as monolayers, in identical medium from which 2-ME had been omitted [10]. Subculturing of these cells was carried out using standard techniques, including trypsinization using 0.25% trypsin/1 mM EDTA. Co-cultures of IMR-90 fibroblasts and Nb2-11 suspension cells were used to show that the fibroblasts, in the absence of cystine uptake enhancers such as 2-ME, could promote replication of the lymphoma cells via secretion of a diffusible factor. To this end, cultures in 12-well tissue culture plates (Linbro; Flow, Mississauga, ON, Canada) of subconfluent IMR-90 fibroblasts (2 ml; around 0.16×10^6 cells/well) were incubated with Nb2-11 cells (1.0 ml; around 50×10^3 cells/ml) contained in culture inserts (0.4 μM pore size, 12-well format; Becton Dickinson, Oakville, ON, Canada). Fischer's medium contains cystine (about 84 μM), approximating cystine concentrations in human plasma, but not cysteine [15].

SASP solutions

As a routine, a 10 mM SASP solution was prepared, under subdued light conditions, by dissolving 40.1 mg of the drug (using a magnetic stirring bar) in 4.0 ml 0.1 N NaOH plus 5.77 ml phosphate-buffered saline (PBS, pH 7.2) and adjusting the pH to about 7.5 by slow addition of 5×50 μl 1.0 N HCl. Using culture medium, appropriate dilutions were then made for immediate addition to cell cultures [15].

Evaluation of growth-inhibitory effects of SASP *in vitro*

Following trypsinization of confluent IMR-90 cell cultures, the fibroblasts were centrifuged (3.5 min at 350 *g*), and resuspended in Fischer's medium supplemented with FBS (10%) and antibiotics. Aliquots (2.0 ml; 60×10^3 cells/ml) were distributed in 12-well Linbro tissue culture plates for a 4-day incubation at 37°C to subconfluency (about 0.25×10^6 cells/well). The supernatants of the cultures were then removed by suction and replaced by either fresh culture medium (1.80 ml) or by suspensions of Nb2-11 cells resuspended in fresh medium free of 2-ME (1.80 ml at about 50×10^3 cells/ml). After a few hours of incubation, SASP, medium or 2-ME was added (final volume 2.0 ml; use of triplicates) for a further 67-h incubation. Cell populations were then determined for the Nb2-11 suspension cells (in the co-cultures) and for trypsinized IMR-90 fibroblasts (cultured in the absence of Nb2-11 cells), using an electronic cell counter (Coulter, Hialeah, FL). The suspended Nb2-11 cells could be readily sampled without significant contamination by fibroblasts, providing the monolayers were intact as observed by microscopy. Culture growth inhibitions were calculated from the cell number

increases found at 67 h in SASP-treated cultures and their controls. Percent inhibition = 100−(increase$_{SASP-treated}$/increase$_{control}$) × 100. It may be noted that the efficacy of SASP *in vitro* was always determined with Fischer's medium that had been freshly prepared from powdered medium.

Testing of SASP efficacy *in vivo*

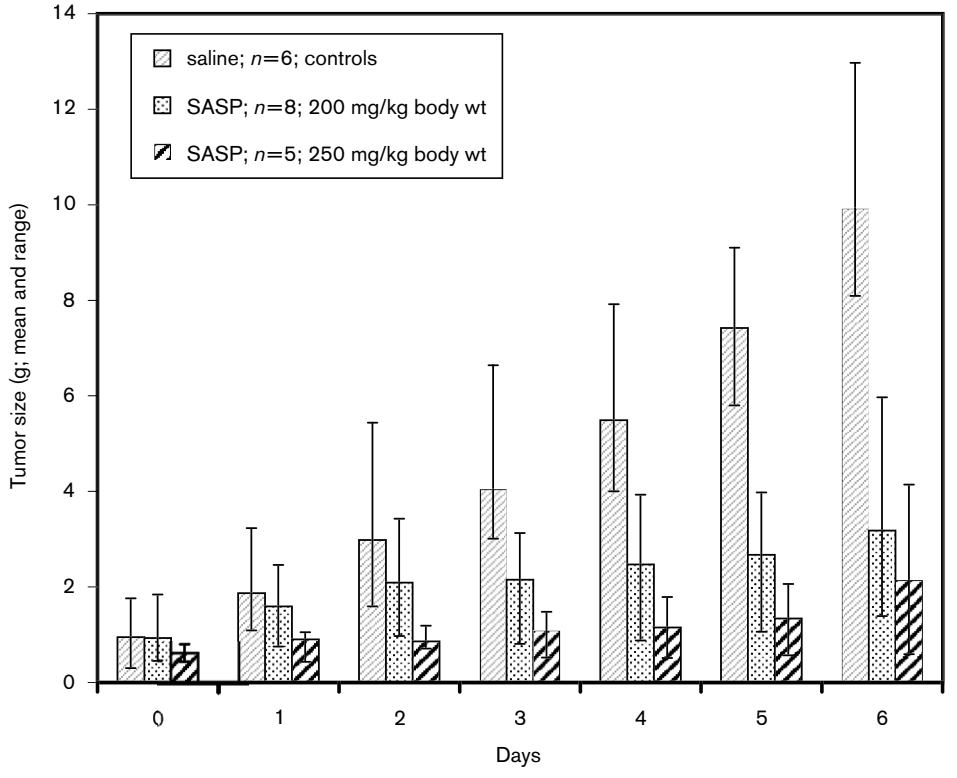
Approximately equal portions of minced Nb2-U17 tumor tissue, developed in a rat following s.c. injection of Nb2-U17 cells, were injected by trocar s.c. in the nape of the neck of groups of male Noble rats (one injection per rat) that had been lightly anesthetized with isoflurane (Abbott, Montreal, PQ, Canada) [15]. When the single tumors reached a measurable size (about 1 cm^3), treatment of the animals (350–400 g) was started using i.p. injections at a site remote from the transplant at approximately 12-h intervals. Fresh SASP solutions (20 mg/ml) were prepared every day in 0.1 N NaOH subsequently adjusted with 1.0 N HCl to a pH of about 8. PBS was used for controls. Drug preparation and administration were carried out under subdued light conditions. Food and water were provided *ad libitum*. Tumor size and health of the rats were monitored daily. Special attention was given to substantial weight loss, prolonged panting, severe diarrhea, hunched posture, unusual behavior, etc., with a view to sacrificing animals if they showed such signs of stress. Tumor size was measured using calipers and expressed in grams using the formula: $\pi/6 \times \text{length} \times \text{width} \times \text{height}$ in cm [17]. Sectioning and histologic analysis of tumor and normal tissues obtained at necropsy were carried out by Criterion Service Laboratory, Vancouver, BC, Canada (pathologist: Dr Jean leRiche, BC Cancer Agency). Animal care and experiments were carried out in strict accordance with the guidelines of the Canadian Council on Animal Care.

Results

Effect of SASP on growth of lymphoma transplants

As part of a previous study [15], the effect of SASP was determined on the growth in male Nb rats of single, well-established, s.c. Nb2-U17 rat lymphoma transplants, previously demonstrated to be non-metastatic [16]. The drug was administered i.p. at well-tolerated dosages starting on day 0, and consecutively every 12 h, in an effort to keep its levels in the circulation high; saline was administered to controls. Presented in Figure 1 are the

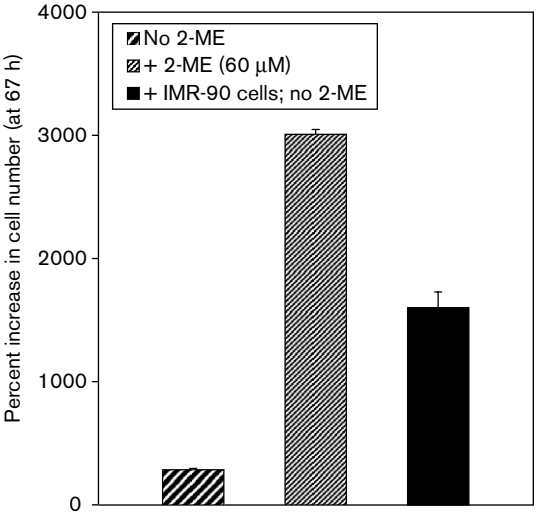
Fig. 1



Effect of treatment with SASP on the growth of well-developed, rapidly growing, s.c. Nb2-U17 lymphoma transplants in Noble rats. Starting on day 0, groups of male rats (350–400 g), each carrying a single tumor in the nape of the neck (range 0.3–1.8 g), received i.p. injections, at 12-h intervals, of saline (controls) or SASP at two dosages, as indicated. Tumor mass (g) was calculated from the length, width and height of the tumors in cm. Each bar represents the average tumor size in a group, with an indication of the smallest and largest tumor size in the group.

effects of SASP on the size of the tumors immediately following drug administration, as distinct from the previous report in which results after 7 days of treatment were presented [15]. The inherent, rapid growth of the Nb2-U17 tumors is demonstrated in the control group, showing an increase in tumor size from an average of about 1 g to an average of about 10 g on day 6. SASP arrested the average growth of the tumors almost completely, in particular at the higher dosage, starting after 1–2 days of treatment. After about 4 days of continuous tumor growth arrest, the size of the tumors in both SASP-treated groups increased slightly on day 6. There was no indication of tumor regression throughout the 7-day period. As reported previously [15], histologic analysis showed no sign of metastasis of the tumor cells to tissues such as the liver, kidney and spleen, i.e. target organs of metastatic Nb2 lymphoma cell lines [16]. This indicated that the suppressive effect of the drug on the size of the Nb2-U17 tumors reflected genuine growth arrest and was not a result of tumor cells escaping from their location. In addition, the treatment with SASP did not induce major toxicity to the hosts, as suggested by lack of the stress signs described above [15].

Fig. 2



2-ME (cysteine) dependence of Nb2-11 lymphoma cells *in vitro*: growth facilitation by IMR-90 fibroblast monolayers in the absence of 2-ME. Suspension cultures of Nb2-11 cells (40×10^3 cells/ml) were incubated at 37°C in the absence of 2-ME, with 2-ME (60 μM) or in co-cultures with subconfluent, cysteine-secreting IMR-90 fibroblast monolayers in the absence of 2-ME. Nb2-11 cell populations were determined after 67 h. The data are representative of results from three experiments.

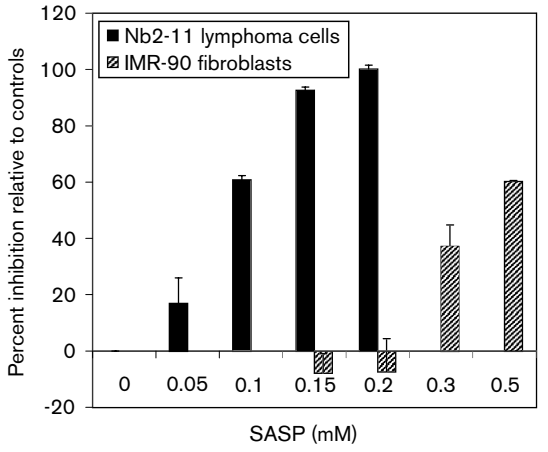
Nb2 lymphoma cell replication supported by IMR-90 fibroblasts in absence of 2-ME

The Nb2-U17 rat lymphoma cell line, and its Nb2-11 subline, have been shown to be dependent for growth and viability on uptake of cyst(e)ine from their micro-environment [7,16]. Both cell lines do not express the x_c^- cystine transporter [15] and have low uptake capability for cystine, i.e. when cultured in Fischer's medium, they require 2-ME for uptake of cystine [7,16]. *In vivo*, the proliferation of these lymphoma cells will therefore be critically dependent on availability in their micro-environment of cysteine at growth-supporting levels, as can be supplied by somatic cells [12,13]. To examine somatic cell-mediated supply of cysteine to lymphoma cells *in vitro*, it was determined whether cells of the widely available Nb2-11 lymphoma cell line could be grown in Fischer's medium/10% FBS, in the absence of 2-ME, by co-culturing them with IMR-90 lung fibroblasts. These cells, like activated macrophages and dendritic cells [12,13], are known to take up cystine via the x_c^- cystine transporter and then secrete thiols, in particular cysteine, into the medium [10]. As shown in Figure 2, the Nb2-11 cells have a significant growth requirement for 2-ME (to mediate uptake of cystine); this requirement could to a large extent be met by the IMR-90 monolayers. Thus the doubling time of the Nb2-11 lymphoma cell cultures, when co-cultured with the fibroblasts, was about 16 h, approximating that of about 13 h regularly obtained for single Nb2-11 cultures in the presence of 60 μM 2-ME [7]. The IMR-90 fibroblasts also allowed replication of Nb2-11 cells in 2-ME-free medium when the lymphoma cells were separated from the monolayers by culturing them in a culture insert (pore size 0.4 μM), restraining cells but not diffusible factors. Under these conditions the lymphoma cells grew actively with a culture doubling time of 26 h, as distinct from no significant growth in the absence of the monolayers. These data indicate that the growth-promoting activity of the IMR-90 fibroblasts was based on secretion of a diffusible factor and did not involve physical interaction with the lymphoma cells. The results, together with observations by other researchers that IMR-90 fibroblasts can promote lymphoma cell proliferation *in vitro* by continuously secreting cysteine [10], strongly indicate that the proliferation of the Nb2-11 lymphoma cells in the co-cultures, in the absence of cystine uptake enhancers, was a result of secretion by the IMR-90 fibroblasts of cysteine.

Growth-inhibitory effect of SASP on fibroblast-driven Nb2-11 cells: prevention by 2-ME

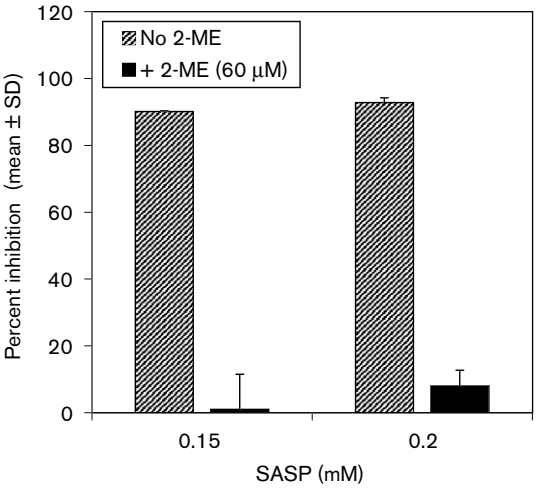
Figure 3 shows the effect of a 67-h incubation with SASP on the proliferation of Nb2-11 cells as promoted, in the absence of 2-ME, by IMR-90 feeder layers. SASP had a very marked inhibitory effect on the proliferation of these x_c^--deficient lymphoma cells. Already at 0.05 mM, SASP showed a growth inhibition of 17%, which rapidly rose to 61, 93 and 100% at 0.1, 0.15 and 0.2 mM SASP, respectively. In sharp contrast, SASP at concentrations

Fig. 3



The effects of a 67-h incubation at 37°C with SASP at increasing concentrations on (i) the proliferation of Nb2-11 cells driven by fibroblasts in co-cultures in the absence of 2-ME and (ii) the proliferation of IMR-90 fibroblasts in single cultures without 2-ME. Details of the experiment are described in Materials and methods. The results are expressed as percentage inhibition relative to control growth (no drug). The data are representative of results from four experiments.

Fig. 4



Inhibition by SASP of fibroblast-driven Nb2-11 lymphoma cell proliferation: prevention by 2-ME. Nb2-11 cells, in co-cultures with IMR-90 fibroblasts, were incubated for 67 h at 37°C with SASP (0.15 and 0.2 mM) in the absence or presence of 2-ME (60 μM). The results are expressed as percentage inhibition relative to control growth (no drug). The data are representative of results from four experiments.

up to and including 0.2 mM had no inhibitory effect on the proliferation of the fibroblasts grown in single cultures. In addition, microscopic observations showed that the fibroblast monolayers after the 67-h treatment with 0.2 mM SASP, whether in single cultures or in co-cultures with the Nb2-11 cells, were completely intact (data not shown). At higher concentrations, however, SASP did affect the proliferation of the fibroblasts, showing growth inhibitions of 37 and 60% at 0.3 and 0.5 mM, respectively. At these SASP concentrations 'gaps' were observed in the fibroblast monolayers (data not shown).

As shown in Figure 4, the inhibitory effects of 0.15 and 0.2 mM SASP on the proliferation of the lymphoma cells, growth-stimulated by IMR-90 fibroblasts in the absence of 2-ME, could almost completely be prevented by including 2-ME (60 μM) in the culture medium. A similar, preventative effect of 2-ME was found for the inhibition by 0.5 mM SASP of the proliferation of the fibroblasts in single cultures (data not shown). 2-ME has been reported to mediate cellular uptake of cystine via the leucine transporter, thus bypassing the x_c^- cystine transporter [18]; it also allows uptake of the amino acid by cells that do not have a specific cystine transporter [15]. The results therefore indicate that the growth arrest of the fibroblast-driven Nb2-11 cells by SASP (0.2 mM or below) was due specifically to intracellular cyst(e)ine deficiency within the lymphoma cells. Since, under the conditions used, the only cysteine available for the growth of the Nb2-11 cells was supplied by the fibroblasts, the arrest of Nb2-11 cell proliferation must have been caused by SASP inhibiting the secretion of cysteine by the fibroblasts, namely by inhibiting their uptake of cystine via the x_c^- cystine transporter. It appears that the fibroblasts, at SASP concentrations in the range 0.1–0.2 mM, were able to take up enough cystine to stay completely viable, but not enough to secrete adequate amounts of cysteine to promote proliferation of the lymphoma cells. At higher SASP concentrations (0.5 mM), the greater inhibition of cystine uptake interfered with the viability of the fibroblasts. This growth requirement of the IMR-90 cells for cystine is consistent with that reported by others [19].

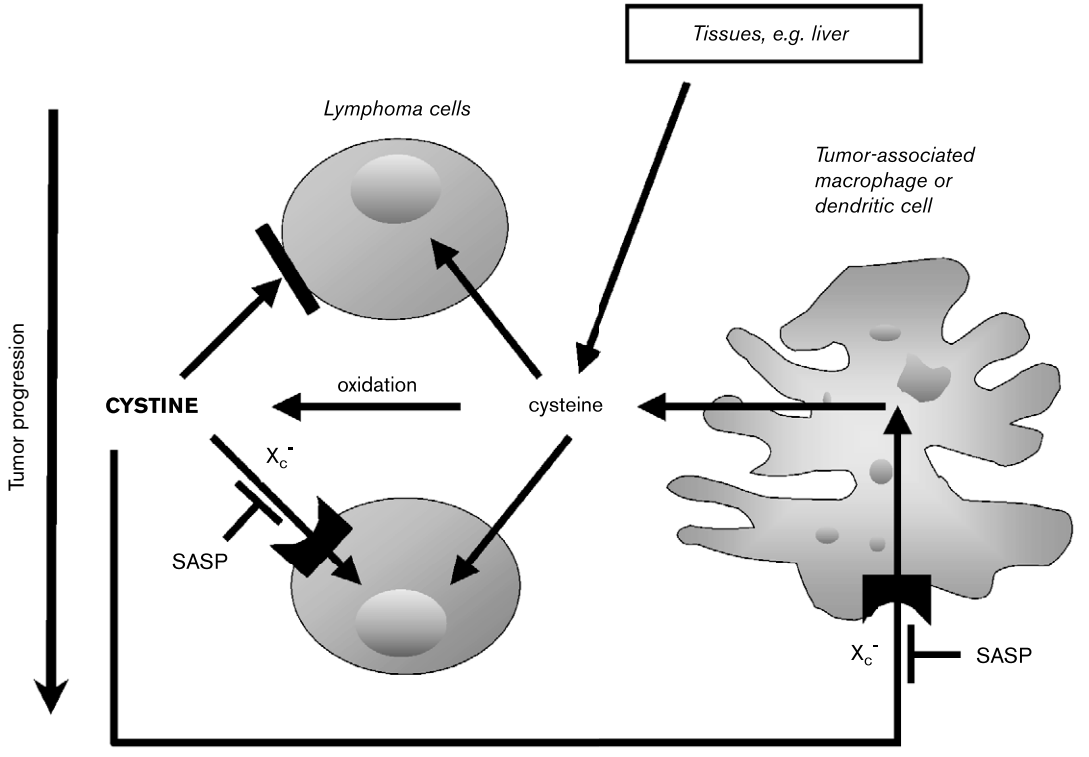
Discussion

Since lymphoid cells require extracellular cyst(e)ine for growth and readily take up cysteine, but in general not cystine, the levels of cysteine in their micro-environment occupy a key position in the regulation of their proliferation. Cysteine is not only removed from the circulation via cellular uptake, but also by rapid oxidation to cystine, which leads to relatively high cystine and very low cysteine levels in the plasma [20]. However, for activation of lymphocytes, cysteine can be supplied by somatic cells via a secretory process mediated by the x_c^- cystine/glutamate antiporter. As reported by various research groups, fibroblasts [10], macrophages [12], monocyte-derived dendritic cells [13] and macrophages [21] can take up cystine via the x_c^- cystine transporter,

reduce it intracellularly and secrete cysteine into their micro-environment. The secretion of cysteine by macrophages appears to be important in the regulation of lymphocyte activation [12]. Similarly, the capacity of activated antigen-presenting cells (e.g. dendritic cells) to release cysteine, following receptor–ligand-based interaction with T lymphocytes, represents a critical parameter for proliferation of the lymphocytes [13], including those of intestinal lamina propria [22]. Monocytes are known to infiltrate tumors, giving rise to tumor-associated macrophages and dendritic cells, which can make up a substantial amount of the tumor mass and, in fact, have been reported to stimulate growth of the cancers by secretion of, for example, cytokines and growth factors [23]. Since lymphoid cancer cells, like lymphocytes, critically require cysteine for growth, the above information suggests that lymphoid cancer-associated macrophages, dendritic cells and fibroblasts could also have an important role in the growth promotion of the cancer cells by supplying them with cysteine. An interference with this process could inhibit the growth of the malignancies. With this in mind the idea was initiated that the x_c^- cystine transporter provides a target for lymphoid cancer therapy [24]. Its inhibition could potentially lead to cyst(e)ine starvation of lymphoid cancers by reducing both the supply of cysteine by tumor-associated somatic cells and the uptake of cystine by cancer cells expressing the x_c^- transporter as a result of tumor progression [7]. It became feasible to investigate this suggestion using SASP, when it was discovered that this drug is a potent inhibitor of the x_c^- cystine transporter, and markedly inhibited lymphoma cell proliferation *in vitro* and growth of lymphoma transplants in rats [15]. A proposed mechanism of action for SASP as an anti-lymphoma agent is presented in Figure 5.

Whereas the arrest of lymphoma cell proliferation by SASP *in vitro* [15] could be readily explained by inhibition of the x_c^- cystine transporter leading to intracellular cyst(e)ine deficiency of the target cells, the marked inhibition by SASP of Nb2-U17 tumor transplant growth in rats (Fig. 1) is not likely due to a direct action of the drug on the tumor cells. Nb2-U17 cells do not express an x_c^- cystine transporter [15] and when such cells are

Fig. 5



Proposed mechanism for SASP-induced cyst(e)ine starvation of lymphoma cells *in vivo*. Circulating cysteine, as produced by, for example, the liver, is readily taken up by lymphoma cells, but also quickly oxidized to cystine, leading to cystine predominance and low levels of cysteine. In contrast to cysteine, cystine is not taken up readily by lymphoma cells unless they express the x_c^- cystine transporter (e.g. as a result of tumor progression). Tumor-associated somatic cells, such as activated macrophages and dendritic cells, can regenerate cysteine from cystine, by taking up cystine via the x_c^- cystine transporter and secreting it as cysteine back into the environment. By inhibiting the x_c^--mediated cystine uptake of the somatic cells, SASP leads to reduced levels of cysteine in the environment of the lymphoma cells; SASP also inhibits uptake of cystine by the advanced lymphoma cells. As a result, the action of SASP may induce cyst(e)ine starvation for both types of lymphoma cells.

adequately supplied with cyst(e)ine, their proliferation is not significantly affected by SASP as shown by the lack of major growth arrest in 2-ME-containing cultures of Nb2-11 cells (Fig. 4) or Nb2-U17 cells, even at a concentration as high as 0.4 mM SASP [15]. Nb2-U17 lymphoma cells, however, have a low uptake capability for cystine [7,16] and hence are expected to critically depend on uptake of extracellular cysteine for growth *in vivo*. Together, these data raise the possibility that the growth arrest of the lymphoma transplants by SASP could have resulted from an interference by the drug with cysteine supply by somatic, tumor-associated cells leading to cysteine starvation of the tumor cells. The results of the present study support this suggestion. Thus, the major arrest of Nb2-11 cell proliferation by 0.15–0.2 mM SASP in the 2-ME-free co-cultures of lymphoma cells and fibroblasts (Fig. 3), was most likely due to interference by SASP with the x_c^--mediated supply by the fibroblasts of cysteine, leading to cysteine starvation of the lymphoma cells. This is indicated by the almost complete prevention of the SASP-induced growth arrest by 60 μM 2-ME, a cystine uptake enhancer (Fig. 4). Importantly, 0.2 mM SASP, a patient-tolerated concentration [25], completely arrested the proliferation of the fibroblast-driven Nb2-11 cells without affecting the replication of the fibroblasts (Fig. 3). This differential is in agreement with the marked arrest of lymphoma growth by SASP in the rats coupled to absence of major toxicity to the hosts (Fig. 1). It may also be noted that it took 1–2 days before treatment with SASP arrested the growth of the lymphoma transplants (Fig. 1), a delay that would be consistent with a gradual development of cysteine and glutathione deficiency within the tumor cells.

Whereas x_c^--mediated uptake of cystine by macrophages, freshly isolated from the mouse peritoneal cavity, is apparently very low, culturing has been reported to induce x_c^- activity leading to a high rate of cystine uptake by such cells, comparable to that of fibroblasts [26]. The latter can secrete cysteine without having to interact physically with lymphoid cells, at least *in vitro*, as indicated in the present study (Fig. 2; text) and as previously reported by others [10]. More recently it has been reported that monocyte-derived dendritic cells, i.e. antigen-presenting cells essential in the development of the immune response, can take up cystine via the x_c^- cystine transporter and secrete cysteine in their environment, in particular following ligand–receptor interaction with T lymphocytes [22] and, especially, alloreactive T cells [13]. Tumors usually contain substantial numbers of macrophages and dendritic cells, some of which are targeted against the tumor cells [23]. It appears very likely therefore that the Nb2-U17 lymphoma transplants used in the present study (Fig. 1) also contained such somatic cells and that the cysteine secreted by the cells, considered essential for lymphocyte activation [13,22], would also promote proliferation of the Nb2 lymphoma cells. In such a scenario, inhibition by SASP of cystine uptake via the x_c^- cystine transporter could lead to an interference with the cysteine secretion by the somatic cells and ultimately to arrest of tumor growth via apoptosis.

With regard to other activities of SASP, it is of interest that this drug has been reported to inhibit interleukin-12 production by lipopolysaccharide-activated macrophages [27], suggesting that it could also inhibit the production/secretion by such cells of other growth factors required for proliferation of lymphoma cells. Nb2-U17 cells, however, have requirements for growth factors which are normally readily available in the circulation. Thus Nb2-U17 cells, like Nb2-11 cells, actively proliferate in chemically defined medium supplemented with 2-ME (60 μM), and prolactin, transferrin, insulin and albumin as the only proteins [28]. It is not likely, therefore, that the anti-lymphoma growth activity of SASP *in vivo* is based on interference with the secretion by somatic cells of polypeptide growth factors.

SASP is also known to inhibit activation of NF-κB, a transcription factor involved in regulation of cell proliferation and differentiation [14]—an inhibitory property that could have played a role in the growth arrest of the lymphoma transplants (Fig. 1). However, we have recently found evidence that SASP can readily inhibit replication of cells *in vitro*, via cyst(e)ine starvation, at concentrations which do not interfere with NF-κB activation. Following up an observation that human MDA-MB-231 breast cancer cells *in vitro* are dependent for growth on uptake of extracellular cystine [29], we have shown that SASP at 0.5 mM completely arrested their replication by cysteine starvation without inhibiting NF-κB activation induced by a 2-h treatment with tumor necrosis factor-α (30 ng/ml) as indicated by electromobility shift assays; significant inhibition of DNA-binding activity of NF-κB was obtained when SASP levels were increased to 5 mM (manuscript in preparation). These observations suggest that the inhibition of Nb2-U17 transplant growth by SASP was not based on inhibition of NF-κB activation, but more likely on an interference with the secretion by somatic cells of cysteine, which can be effective at SASP concentrations as low 0.15 mM (Fig. 3).

Glutathione, a major scavenger of free radicals, plays an important role in the defense of cells against oxidative stress and drug resistance of cancers is frequently associated with increases in their rates of glutathione synthesis [30]. The synthesis of glutathione is critically dependent on intracellular levels of cysteine [1] which, in lymphoid cells, are subject to cellular uptake of cyst(e)ine. With regard to this, it is notable that Nb2-SFJCD1 rat lymphoma cells, in contrast to the closely

related Nb2-U17 and Nb2-11 lymphoma cells [16], display an enhanced capacity for producing glutathione when challenged with oxidative stress and show a significantly higher resistance to H_2O_2-induced apoptosis [31]. This property is presumably a reflection of the fact that this cell line, as distinct from the other two, expresses the x_c^- cystine transporter [15], thereby greatly enhancing its capacity for uptake of extracellular cystine required for glutathione synthesis [1,7]. It may also be noted that SASP, by inhibiting cellular uptake of cystine, leads to reduced levels of intracellular glutathione, as recently shown with MDA-MB-231 cells [29] and as expected on the basis of a decrease in intracellular cyst(e)ine levels [3,4]. In view of the above, it appears that SASP will also be useful for reducing glutathione-based resistance developed by cancer cells against treatment with free radical-generating drugs. The inhibitory effect of SASP on glutathione-S-transferases, enzymes involved in protection of cells from oxidative stress [32], would similarly be useful in chemotherapy using SASP in combination with, for example, free radical-generating alkylating drugs.

In addition to lymphoid cancers, SASP could also affect the growth of other malignancies that depend on uptake of extracellular cyst(e)ine for growth. As previously reported, these may include certain cancers of neuronal tissue and skin [15]. Recently, cultures of human breast cancer cells, such as the MDA-MB-231 cells, were found to depend on extracellular cyst(e)ine for growth and to respond to SASP [29]. In addition we found that the related MDA-MB-468 breast cancer cell line, although growing relatively slowly in Fischer's medium supplemented with 10% FBS (doubling time = 35–40 h), is as sensitive to treatment with SASP as the rat Nb2-SFJCD1 lymphoma cell line (doubling time = 13 h) previously studied [15] (Doxsee and Gout, unpublished data). This raises the possibility that growth of tumors of such breast cancer cells in immuno-deficient mice may also respond to treatment with SASP.

Oral administration of SASP, commonly used in the treatment of rheumatoid arthritis and inflammatory bowel disease, leads to major degradation of the drug; it is reduced by intestinal bacteria via azo cleavage to sulfapyridine and 5-aminosalicylic acid [14]. These colonic metabolites have no x_c^- inhibitory activity [15] and SASP, when used to target the x_c^- cystine transporter, should therefore not be administered orally. In this regard it is of interest that lymphoid follicular proctitis, a condition of the rectal mucosa characterized by excessive numbers of lymphocytes, can be successfully treated using SASP suppositories in contrast to oral administration of SASP [33]. This difference in drug efficacy may be related to greater stability of SASP when administered as a suppository as distinct from an oral formulation. Since the proliferation of intestinal lamina propria T lymphocytes can apparently be regulated by cysteine secreted by activated antigen-presenting cells [22], the effectiveness of the treatment with SASP suppositories may be based on interference by intact SASP with the cysteine secretion by the monocytes. With regard to the use of SASP as an anticancer drug it is of major interest that azo cleavage of orally administered SASP appears to be markedly reduced in patients taking antibiotics [34]. This suggests that oral, short-term administration of SASP against malignancies such as lymphoid cancers may be effective when used in combination with antibiotics.

Conclusion

Evidence has been obtained that SASP at certain concentrations can inhibit proliferation of lymphoma cells by interfering with the cysteine supply by somatic cells, without affecting the viability of the somatic cells. Such action may underlie the marked arrest by SASP of lymphoma transplant growth in rats without major side effects to the hosts.

Acknowledgments

Dr Charles T. Beer is thanked for helpful discussions and Dan Doxsee for excellent technical assistance.

References

1 Meister A. Glutathione, ascorbate, and cellular protection. *Cancer Res* 1994; **54**:1969s–1975s.
2 Hutter DE, Till BG, Greene JJ. Redox state changes in density-dependent regulation of proliferation. *Exp Cell Res* 1997; **232**:435–438.
3 Yonezawa M, Back SA, Gan X, Rosenberg PA, Volpe JJ. Cystine deprivation induces oligodendroglial death: rescue by free radical scavengers and by a diffusible glial factor. *J Neurochem* 1996; **67**:566–573.
4 Iwata S, Hori T, Sato N, Hirota K, Sasada T, Mitsui A, *et al.* Adult T cell leukemia (ATL)-derived factor/human thioredoxin prevents apoptosis of lymphoid cells induced by L-cystine and glutathione depletion. *J Immunol* 1997; **158**:3108–3117.
5 Iglehart JD, York RM, Modest AP, Lazarus H, Livingston DM. Cystine requirement of continuous human lymphoid cell lines of normal and leukemic origin. *J Biol Chem* 1977; **252**:7184–7191.
6 Uren JR, Lazarus H. L-Cyst(e)ine requirements of malignant cells and progress toward depletion therapy. *Cancer Treat Rep* 1979; **63**:1073–1079.
7 Gout PW, Kang YJ, Buckley DJ, Bruchovsky N, Buckley AR. Increased cystine uptake capability associated with malignant progression of Nb2 lymphoma cells. *Leukemia* 1997; **11**:1329–1337.
8 Gmünder H, Eck H-P, Dröge W. Low membrane transport activity for cystine in resting and mitogenically stimulated human lymphocyte preparations and human T cell clones. *Eur J Biochem* 1991; **201**:113–117.
9 Broome JD, Jeng MW. Promotion of replication in lymphoid cells by specific thiols and disulfides *in vitro*. *J Exp Med* 1973; **138**:574–592.
10 Ishii T, Hishinuma I, Bannai S, Sugita Y. Mechanism of growth promotion of mouse lymphoma L1210 cells *in vitro* by feeder layer or 2-mercaptoethanol. *J Cell Physiol* 1981; **107**:283–293.
11 Bannai S. Exchange of cystine and glutamate across plasma membrane of human fibroblasts. *J Biol Chem* 1986; **261**:2256–2263.
12 Gmünder H, Eck H-P, Benninghoff B, Roth S, Dröge W. Macrophages regulate intracellular glutathione levels of lymphocytes. Evidence for an immunoregulatory role of cysteine. *Cell Immunol* 1990; **129**:32–46.
13 Angelini G, Gardella S, Ardy M, Ciriolo MR, Filomeni G, Di Trapeni G, *et al.* Antigen-presenting dendritic cells provide the reducing extracellular microenvironment required for T lymphocyte activation. *Proc Natl Acad Sci USA* 2002; **99**:1491–1496.
14 Wahl C, Liptay S, Adler G, Schmid RM. Sulfasalazine, a potent and specific inhibitor of nuclear factor kappa B. *J Clin Invest* 1998; **101**:1163–1174.

15 Gout PW, Buckley AR, Simms CR, Bruchovsky N. Sulfasalazine, a potent suppressor of lymphoma growth by inhibition of the x_c^- cystine transporter: a new action for an old drug. *Leukemia* 2001; **15**:1633–1640.
16 Gout PW, Horsman DE, Fox K, de Jong G, Ma S, Bruchovsky N. The rat Nb2 lymphoma: a novel model for tumor progression. *Anticancer Res* 1994; **14**:2485–2492.
17 Tomayko MM, Reynolds CP. Determination of subcutaneous tumor size in athymic (nude) mice. *Cancer Chemother Pharmacol* 1989; **24**:148–154.
18 Ishii T, Bannai S, Sugita Y. Mechanism of growth stimulation of L1210 cells by 2-mercaptoethanol *in vitro*. Role of the mixed disulfide of 2-mercaptoethanol and cysteine. *J Biol Chem* 1981; **256**:12387–12392.
19 Aoshiba K, Yasui S, Nishimura K, Nagai A. Thiol depletion induces apoptosis in cultured lung fibroblasts. *Am J Respir Cell Mol Biol* 1999; **21**:54–64.
20 Mansoor MA, Svardal AM, Ueland PM. Determination of the *in vivo* redox status of cysteine, cysteinylglycine, homocysteine, and glutathione in human plasma. *Anal Biochem* 1992; **200**:218–229.
21 Rimaniol A-C, Mialocq P, Clayette P, Dormont D, Gras G. Role of glutamate transporters in the regulation of glutathione levels in human macrophages. *Am J Physiol Cell Physiol* 2001; **281**:C1964–C1970.
22 Sido B, Braunstein J, Breitkreutz R, Herfarth C, Meuer SC. Thiol-mediated redox regulation of intestinal lamina propria T lymphocytes. *J Exp Med* 2000; **192**:907–912.
23 Seljelid R, Jozefowski S, Sveinbjornsson B. Tumor stroma. *Anticancer Res* 1999; **19**:4809–4822.
24 Gout PW. Progression of Nb2 lymphoma cells from hormonal dependency: association with increased cystine uptake via the x_c^- transport system. *Proc 79th Annu Endocr Soc Meet* 1997; 3–16.
25 Guastavino E, Litwin NH, Heffes Nahmod L, Licastro R. Ulcerative colitis in children. Levels of salicylazosulfapyridine and sulfapyridine during treatment. *Acta Gastroenterol Latinoam* 1988; **18**:107–113.
26 Watanabe H, Bannai S. Induction of cystine transport activity in mouse peritoneal macrophages. *J Exp Med* 1987; **165**:628–640.
27 Hasko G, Szabo C, Nemeth ZH, Deitch EA. Sulphasalazine inhibits macrophage activation: inhibitory effects on inducible nitric oxide synthase expression, interleukin-12 production and major histocompatibility complex II expression. *Immunology* 2001; **103**:473–478.
28 Gilks CB, Porter SD, Barker C, Tsichlis PN, Gout PW. Prolactin (PRL)-dependent expression of a zinc finger protein-encoding gene, Gfi1, in Nb2 lymphoma cells: constitutive expression in autonomous sublines. *Endocrinology* 1995; **136**:1805–1808.
29 Narang VS, Buckley DJ, Pauletti GM, Gout PW, Buckley AR. Increased reliance on the x_c^- cystine/glutamate antiporter with malignant progression of breast cancer. *Proc Am Ass Cancer Res* 2002; **43**:2918.
30 Gamesik MP, Dubay GR, Cox BR. Increased rate of glutathione synthesis from cystine in drug-resistant MCF-7 cells. *Biochem Pharmacol* 2002; **63**:843–851.
31 Meyer TM, Liang HQ, Buckley AR, Buckley DJ, Gout PW, Green EH, *et al.* Changes in glutathione redox cycling and oxidative stress response in the malignant progression of Nb2 lymphoma cells. *Int J Cancer* 1998; **77**: 55–63.
32 Bach MK, Brashler JR, Johnson MA. Inhibition by sulfasalazine of LTC synthetase and of rat liver glutathione S-transferases. *Biochem Pharmacol* 1985; **34**:2695–2704.
33 Toyoda H, Yamaguchi M, Uemura Y, Mukai K, Sawa H, Suzuki H, *et al.* Successful treatment of lymphoid follicular proctitis with sulfasalazine suppositories. *Am J Gastroenterol* 2000; **95**:2403–2404.
34 Klotz U. Clinical pharmacokinetics of sulphasalazine, its metabolites and other prodrugs of 5-aminosalicylic acid. *Clin Pharmacokinet* 1985; **10**: 285–302.