

Ammonium Trichloro(dioxoethylene-*o,o'*)tellurate (AS101) Sensitizes Tumors to Chemotherapy by Inhibiting the Tumor Interleukin 10 Autocrine Loop

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ABSTRACT

Cancer cells of different solid and hematopoietic tumors express growth factors in respective stages of tumor progression, which by autocrine and paracrine effects enable them to grow autonomously. Here we show that the murine B16 melanoma cell line and two human primary cultures of stomach adenocarcinoma and glioblastoma multiforme (GBM) constitutively secrete interleukin (IL)-10 in an autocrine/paracrine manner. This cytokine is essential for tumor cell proliferation because its neutralization decreases clonogenicity of malignant cells, whereas addition of recombinant IL-10 increases cell proliferation. The immunomodulator ammonium trichloro(dioxoethylene-*o,o'*)tellurate (AS101) decreased cell proliferation by inhibiting IL-10. This activity was abrogated by exogenous addition of recombinant IL-10. IL-10 inhibition by AS101 results in dephosphorylation of Stat3, followed by reduced expression of Bcl-2. Moreover, these activities of AS101 are associated with sensitization of tumor cells to chemotherapeutic drugs, resulting in their increased apoptosis. More importantly, AS101 sensitizes the human aggressive GBM tumor to paclitaxel both *in vitro* and *in vivo* by virtue of IL-10 inhibition. AS101 sensitizes GBM cells to paclitaxel at concentrations that do not affect tumor cells. This sensitization can also be obtained by transfection of GBM cells with IL-10 antisense oligonucleotides. Sensitization of GBM tumors to paclitaxel (Taxol) *in vivo* was obtained by either AS101 or by implantation of antisense IL-10-transfected cells. The results indicate that the IL-10 autocrine/paracrine loop plays an important role in the resistance of certain tumors to chemotherapeutic drugs. Therefore, anti-IL-10 treatment modalities with compounds such as AS101, combined with chemotherapy, may be effective in the treatment of certain malignancies.

INTRODUCTION

Malignant cells are equipped with a number of countermeasures, allowing them to escape immune surveillance. Among the many strategies shared by cancer cells of different solid and hematopoietic tumors, the production and secretion of soluble factors capable of inhibiting specific antitumor immune responses appear to be major mechanisms. Other potential strategies used by tumors to avoid host immune responses include down-regulation of MHC molecules, expression of poorly immunogenic tumor antigens, masking or shedding of immunogenic tumor antigens, and induction of tolerance via display of an incomplete antigen-presenting function (1, 2). Cytokines can be produced by tumor and/or reactive cells, and their secretion can provide a growth advantage for tumor cells in either an autocrine or a paracrine fashion.

Evidence has accumulated that cancer patients have an imbalance

in the T helper 1/T helper 2 (TH1/TH2) axis, with the latter predominating over the former (2). IL-10 is spontaneously secreted by a variety of human cancer cells, including lymphoma, ovarian carcinoma, melanoma (3), neuroblastoma, renal cell carcinoma (4), colon carcinoma (5), glioma (6), and non-small cell lung cancer (7). IL-10 was initially identified as a factor produced by T helper 2 cells that inhibits cytokine synthesis by TH1 cells, an effect attributed to inhibition of the accessory function of macrophages, including down-regulation of MHC class II expression, leading to impaired antigen presentation to reactive T cells (8). Recently, it has been shown that transforming growth factor β may help shift the TH1/TH2 balance toward TH2 by favoring the development of TH2-type cells via IL-10 overproduction and the inhibition of TH1-type responses (9). Not surprisingly, high IL-10 levels have been detected in sera from patients with a wide variety of solid and hematopoietic tumors (7, 10, 11). Elevated levels of IL-10 were observed before surgery. Interestingly, IL-10 serum levels in colon cancer patients appeared to be better screening markers for cancer than conventional tumor markers: levels of the former were in fact found to be abnormally elevated in most patients, whereas levels of the latter were observed within the normal range in a substantial percentage of patients; moreover, IL-10 serum levels were also found to correlate with tumor progression, the presence of distant metastases, and high values of lactate dehydrogenase (5). Most importantly, the curative and noncurative surgery rates were correctly predicted by preoperative IL-10 serum levels in the majority of cases, with high cytokine serum levels paralleled by low rates of curative surgery (5).

Recently, B16 melanoma cells transfected with IL-10 cDNA have been demonstrated to develop into tumors with a higher volume in the syngeneic C57BL/6 mouse, which directly correlated with the amount of IL-10 secreted by tumor cells. The increase of tumor growth by IL-10 was induced by three different simultaneous mechanisms: direct stimulation of cell proliferation through an autocrine mechanism, induction of angiogenesis, and the suppression of the local immune response (12).

On binding of IL-10 to the IL-10 receptor, receptor-associated Janus activated kinase (Jak) tyrosine kinases are activated and stimulate downstream signaling. Jak1, TYK2, and signal transducer and activator of transcription 3 (Stat3) are all involved in this signaling cascade. Activated Stat3 protein translocates to the nucleus and regulates specific gene expression (13). Considerable evidence suggests that constitutive activation of Stat3 actively participates in tumor formation and progression, and many studies have contributed to delineation of the mechanisms underlying persistent, oncogenic Stat3 signaling in tumor cells (14).

The nontoxic immunomodulator ammonium trichloro(dioxoethylene-*o,o'*)tellurate (AS101), first developed by us, is a white crystalline, synthetic organic tellurium compound. The empirical formula is $C_2H_8O_2NCl_3Te$ with a molecular weight of ~ 312 . AS101 was synthesized by refluxing tellurium chloride and ethylene glycol in acetonitrile. It is soluble in organic solvents but only slightly soluble in

Received 10/27/03; revised 12/15/03; accepted 1/7/04.

Grant support: Israel Cancer Association Grant 20030001-B, a Project Grant from the Israel Cancer Research Fund, The Milton Shiffman Global Research Program, the Dorsha Wallman Cancer Research Endowment, and the Frieda Stollman Cancer Memorial Fund.

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water. AS101 has been shown to have beneficial effects in diverse preclinical and clinical studies. Most of its activities have been primarily attributed to the direct inhibition of the anti-inflammatory cytokine interleukin (IL)-10 (15), followed by the simultaneous increase of specific cytokines (16–18). These immunomodulatory properties were found to be crucial for the clinical activities of AS101, demonstrating the protective effects of AS101 in parasite- and virus-infected mice models (19), in autoimmune diseases (20), in septic mice (21), and in a variety of tumor models in mice and humans where AS101 had a clear antitumoral effect (16, 22). Phase I clinical trials with advanced cancer patients treated with AS101 showed increased production and secretion of a variety of cytokines, leading to a clear dominance in TH1 responses with a concurrent decrease in the TH2 responses (22). The predominance of TH1 responses was shown to be related to AS101 antitumoral activity. All AS101 activities were associated with minimal toxicity. AS101 was shown to protect mice from hematopoietic damage caused by sublethal doses of chemotherapy and increased the rate of survival of mice treated with different cytotoxic drugs acting by distinct mechanisms (17, 23, 24). In light of these results, Phase II clinical trials with cancer patients treated with AS101 in combination with chemotherapy have been initiated and completed, showing that treatment with AS101, with no major toxicity, induced a significant reduction in the severity of neutropenia and thrombocytopenia that accompany chemotherapy (25). Phase II clinical studies on systemically treated advanced cancer patients and topically treated virus-infected patients have been initiated recently. In view of the central role of IL-10 inhibition in most of the preclinical activities of AS101, this study is designed to delineate the ability of this tellurium compound to disrupt the IL-10 autocrine/paracrine loop in a variety of tumor cells and to define the resulting signaling and therapeutic implications of this effect.

MATERIALS AND METHODS

Reagents. The following were used: anti-mouse and anti-human IL-10 neutralizing IgG (R&D Systems, Minneapolis, MN), anti-Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA), human and mouse recombinant IL (rIL)-10 (R&D Systems), anti-pStat3 and anti-Stat3 (New England Biolabs, Beverly, MA), paclitaxel (Bristol-Myers Squibb, Syracuse, NY), 5-fluorouracil (Amersham Pharmacia Biotech, Uppsala, Sweden), and doxorubicin (Sigma Aldrich, Rehovot, Israel). AS101 was supplied by Prof. M. Albeck from the Chemistry Department of Bar-Ilan University in a solution of PBS (pH 7.4) and maintained at 4°C.

Animals. Male severe combined immunodeficient (SCID) mice of BALB/c origin, 6–8 weeks of age, were purchased from Harlan Laboratories, Israel. Animal experiments were performed in accordance with approved institutional protocols and approved by the Institutional Animal Care and Use Committee.

Cell Cultures. Three cell cultures were used: an established cell line of murine origin and two primary tumor cell cultures of human origin. Informed consent of the patients was obtained and approved by the local ethics committee. Tumor samples were obtained intraoperatively from nonnecrotic parts of a tumor and were confirmed histopathologically. Tumor biopsy specimens were finely minced and maintained in RPMI 1640 (Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with heat inactivated 20% FCS (Biological Industries), 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (Biological Industries). The cell cultures used in the study were the B16-F10 (B16) murine cell line of C57BL/6 origin, the human stomach adenocarcinoma, and human glioblastoma multiforme (GBM).

Three primary cultures of stomach adenocarcinoma and two of GBM were successfully maintained for >20 passages, and all of them secreted high amounts of IL-10. The data summarized in the study refer to one culture of each type tested on passages 6–20. 100% of GBM cells stained positive for glial fibrillary acidic protein. >95% of culture cells derived from stomach adenocarcinoma stained positive for low molecular weight cytokeratin and carcinoembryonic antigen.

Transfection of Antisense Oligonucleotides (ODNs). Phosphothioate-modified antisense or mismatch control ODNs were purchased from MWG-Biotech AG (Ebersberg, Germany) and dissolved in water. The ODNs directed against the human IL-10 gene product had the following sequences: antisense IL-10, 5'-CTG GGT CAG CTA TCC CAG-3'; and control to antisense (AS) IL-10, 5'-CTG GGA TAG CTG ACC CAG-3'.

For transfection of ODNs, polycationic transfection reagent (Lipofectamine; Invitrogen, Carlsbad, CA) was used to facilitate uptake of ODNs, according to the protocol recommended by the manufacturer. A mixture of ODNs with polycationic transfection reagent was preincubated with cells without serum for 3 h and an additional 24 h with serum. For assessment of transfection efficiency, cell lysates were prepared and analyzed by Western blot analysis for human IL-10 protein expression.

In Vivo Treatment Protocol. SCID mice were unilaterally and s.c. injected with 5×10^6 cultured GBM cells on day 0. Mice were treated as follows:

Daily injection with PBS ($n = 10$).

Daily i.p. injection with AS101 (0.5 mg/kg) starting 1 day after the tumor was palpable (day 25) until the end of the experiment (AS101; $n = 10$).

i.p. injections of paclitaxel on alternate days at 17 mg/kg, starting on day 26, with a total of four paclitaxel (Paclitaxel) injections ($n = 10$).

Daily i.p. injections with AS101 (0.5 mg/kg) and paclitaxel (17 mg/kg) for four treatments (AS101 + Taxol; $n = 10$).

Daily s.c. injections with human rIL-10 (2.25 µg/injection), starting on day 25 (human IL-10; $n = 11$).

Daily injections of AS101 and human rIL-10, starting on day 25, and four paclitaxel injections on alternate days, starting on day 26 (human IL-10 + paclitaxel + AS101; $n = 11$).

Daily injections of PBS to mice inoculated with 5×10^6 GBM cells transfected with control ODNs (control oligo; $n = 10$).

Injections of paclitaxel on alternate days at 17 mg/kg, starting on day 26, with a total of four paclitaxel injections to mice inoculated with 5×10^6 GBM cells transfected with control ODNs (control oligo + paclitaxel; $n = 10$).

Daily injections of PBS to mice inoculated with 5×10^6 GBM cells transfected with IL-10 antisense ODNs (antisense IL-10; $n = 10$).

Injections of paclitaxel on alternate days starting on day 26, with a total of 4 injections, to mice inoculated with 5×10^6 of GBM cells transfected with IL-10 antisense ODNs (antisense IL-10 + paclitaxel) ($n = 10$).

Daily i.p. injections with PBS to control mice not inoculated with GBM cells (healthy; $n = 3$).

Mice were checked three times weekly for their rate of survival and their tumor weight. Tumors were measured with a caliper, and their weight (mg) was estimated from two-dimensional tumor measurements (mm).

$$\text{Tumor weight} = \frac{\text{length} \times \text{width}^2}{2}$$

On day 42, three mice from each group were sacrificed, sera were collected for the evaluation of IL-10 content, and tumors were dissected for preparation of cell lysates.

Clonogenic Assay. Cells were counted and plated at 1000 cells/plate in RPMI 1640 supplemented with 10% FCS and various doses of specific compounds. They were then incubated for macroscopic colony formation. After 8 to 9 days of incubation, colonies were fixed with methanol, stained with Giemsa, and counted if the colonies contained >50 cells.

Detection of Apoptosis. The percentage of cells undergoing apoptosis was quantitatively determined using an apoptosis detection kit (R&D) by virtue of their ability to bind Annexin V and exclude propidium iodide.

Quantitation of IL-10. Cells were cultured at various doses for different time points with or without AS101. Supernatants were collected and evaluated by ELISA kits (R&D).

Western Blot Analysis. Total cell extracts were prepared by suspension in ice-cold lysis buffer containing 50 mM Tris (pH 8), 150 mM NaCl, 1% NP40, 0.1% SDS, 5 mM EDTA, 0.5% deoxycholate, 0.2 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 200 µM sodium vanadate, 5 µg/ml aprotinin, and 5 µg/ml leupeptin. Cell lysates were boiled and electrophoresed on 10% SDS-PAGE and were then blotted with specific antibodies. Blots were developed using horseradish peroxidase-conjugated secondary antibodies and the ECL detection system (Amersham Pharmacia Biotech).

Statistical Analysis. Data are presented as mean ± SE. For comparisons of means of the various groups in the *in vitro* studies, the pairwise *t* test was used. For comparisons between groups in the *in vivo* studies and in some of the *in vitro* studies, we used ANOVA followed by *t* test for multiple comparisons. Survival curves were statistically analyzed by comparing the cumulative percentage of survival using the Gehan-Wilcoxon test. Two-tail *P* < 0.05 was considered significant.

RESULTS

IL-10 Is an Autocrine Growth Factor for Certain Mouse and Human Tumor Cells. Fig. 1 shows that both B16 melanoma cells and the human primary cultures of either stomach adenocarcinoma or GBM constitutively secrete considerable amounts of IL-10 in a time-dependent manner. Optimal IL-10 production is obtained at 24–48 h. At 48 h, IL-10 levels amounted to 272 ± 90 pg/ml (B16), 240 ± 23 pg/ml (stomach adenocarcinoma), and 460 ± 41 pg/ml (GBM). Secretion of IL-10 was gradually and significantly decreased at 72 and 96 h (*P* < 0.01).

The ability of IL-10 to accelerate the proliferation of mouse and

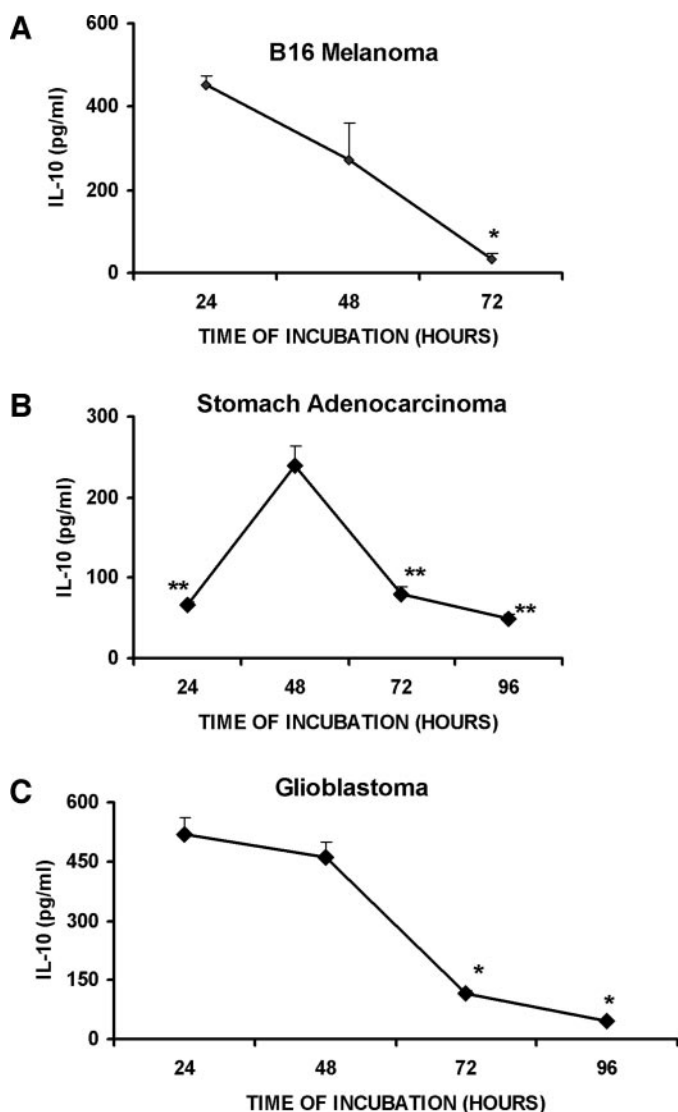


Fig. 1. Autocrine secretion of IL-10 by tumor cells *in vitro*. One × 10⁶ B16 melanoma cells (A), stomach adenocarcinoma cells (B), or GBM cells (C) were cultured in 4 ml of medium for various time points. Supernatants were collected and evaluated for IL-10 content. *, *P* < 0.01 decrease versus 24 h. **, *P* < 0.01 decrease versus 48 h. The results represent the means from three different experiments; bars, SE.

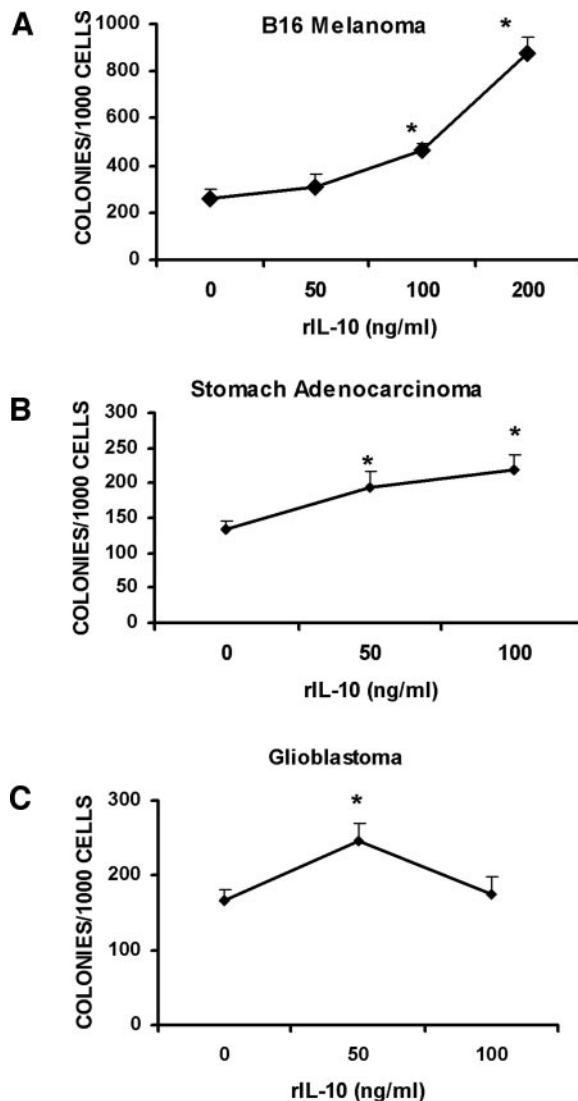


Fig. 2. IL-10 induces proliferation of tumor cells *in vitro*. One × 10³ B16 melanoma cells (A), stomach adenocarcinoma cells (B), or GBM cells (C) were cultured in 4 ml of medium in the presence of various recombinant murine IL-10 (A) or recombinant human IL-10 (B and C). Clonogenicity of tumor cells was assessed. *, *P* < 0.01 increase versus zero. The results represent means from three different experiments; bars, SE.

human tumor cells is illustrated in Fig. 2. IL-10 promotes tumor cell proliferation in a dose-dependent manner, as reflected by their increased clonogenicity. At 50 ng/ml of IL-10, 194 ± 23 and 246 ± 24 colonies developed from stomach adenocarcinoma and GBM cells, respectively, as compared to 134 ± 12 and 165.5 ± 16 in control cells (*P* < 0.01). In B16 melanoma cells, 100 ng/ml IL-10 promoted the increase in clonogenicity from 263 ± 40 to 468 ± 23 (*P* < 0.01), whereas at 200 ng/ml, a 3.5-fold increase was obtained (Fig. 2).

The dependency of tumor cell proliferation on IL-10 could be deduced from the pronounced inhibition of clonogenicity when IL-10 was neutralized by anti-IL-10 neutralizing antibodies. This effect was significant at all tumor cultures but was most prominent in B16 melanoma cells. In these cell cultures, addition of 1 μg/ml anti-IL-10 abs resulted in 85% inhibition of clonogenicity, decreasing the number of colonies from 208 ± 12.4 to 32 ± 25 (*P* < 0.01). At 10 μg/ml, almost complete inhibition occurred in all tumor cell cultures (Fig. 3).

The Immunomodulator AS101 Reduces Clonogenicity of Tumor Cells Via Inhibition of IL-10. We used AS101 as an anti-IL-10 compound to find whether this property will enable it to inhibit the proliferation of tumor cells. Fig. 4A shows that AS101 decreases

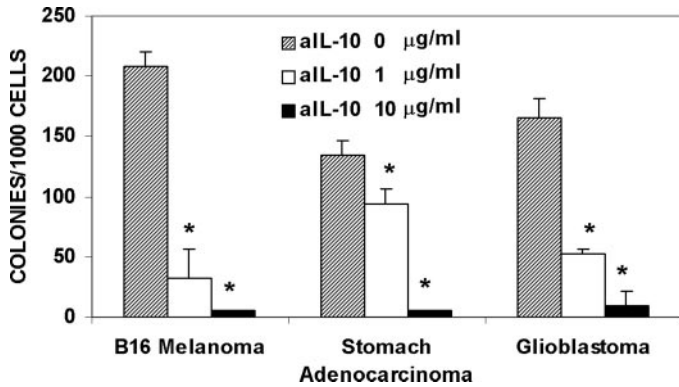


Fig. 3. Neutralization of IL-10 attenuates tumor cell proliferation *in vitro*. One thousand tumor cells were cultured in Petri dishes containing 4 ml of medium and various doses of anti-IL-10 neutralizing antibodies. Clonogenicity of tumor cells was assessed. *, $P < 0.01$ decrease versus zero. The results represent means from three different experiments; bars, SE.

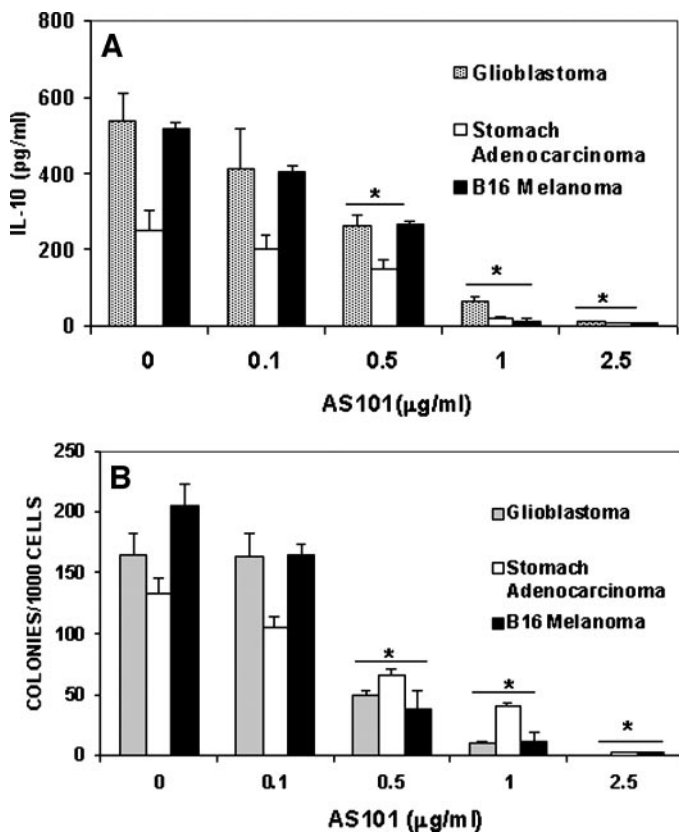


Fig. 4. AS101 inhibits autocrine IL-10 secretion and proliferation of tumor cells *in vitro*. AS101 at various concentrations was introduced to tumor cell cultures. Secretion of IL-10 (A) and clonogenicity (B) were assessed. *, $P < 0.01$ decrease versus zero. The results represent means from three different experiments; bars, SE.

IL-10 production in a dose-dependent manner by both murine and human tumor cells. This decrease is significant starting from 0.5 to 2.5 μg/ml ($P < 0.01$). Incubation of B16 melanoma, stomach adenocarcinoma, and GBM cells in the presence of AS101 gradually and significantly decreased tumor cell proliferation (Fig. 4B). This inhibitory activity of AS101 was abrogated in the presence of rIL-10 in a dose-dependent manner (Fig. 5), suggesting that inhibition of proliferation by AS101 may be attributable to the inhibition of IL-10.

IL-10 Induces Phosphorylation of Stat3 in B16 Melanoma Cells. To gain further insight into the mechanism of IL-10-induced increased proliferation in tumor cells and the consequences of its

inhibition by AS101, we chose the B16 melanoma cell line as a model. The best characterized IL-10 signaling pathway is the Jak/Stat system. The IL-10/IL-10R interaction engages the Jak family tyrosine kinases Jak1 and Tyk2 and induces tyrosine phosphorylation and activation of the latent transcription factors Stat3, Stat1, and Stat5. However, Stat3 is used primarily by IL-10 to carry out its signaling into the cell (13). To ascertain that Stat3 is phosphorylated in B16 melanoma cells, IL-10 was added to starved B16 melanoma cells at various time points. As can be seen in Fig. 6A, IL-10 at 50 ng/ml substantially increased the phosphorylation of Stat3 in a time-dependent manner. The most prominent phosphorylation was detected after 5 and 10 min of incubation with IL-10, whereas at 15min, this activity significantly decreased (Fig. 6A)

Treatment with AS101 or Neutralization of IL-10 Inhibit Stat3 Phosphorylation. B16 melanoma cells, like many other tumor cells, constitutively express activated Stat3. This is reflected by the abundant expression of phosphorylated Stat3 by B16 cells cultured in complete medium containing serum (Fig. 6B). To explore the possibility that Stat3 is constitutively activated in B16 cells by virtue of the autocrine/paracrine IL-10 loop and to assess whether AS101 can deactivate this transcription factor by interrupting this loop, we first

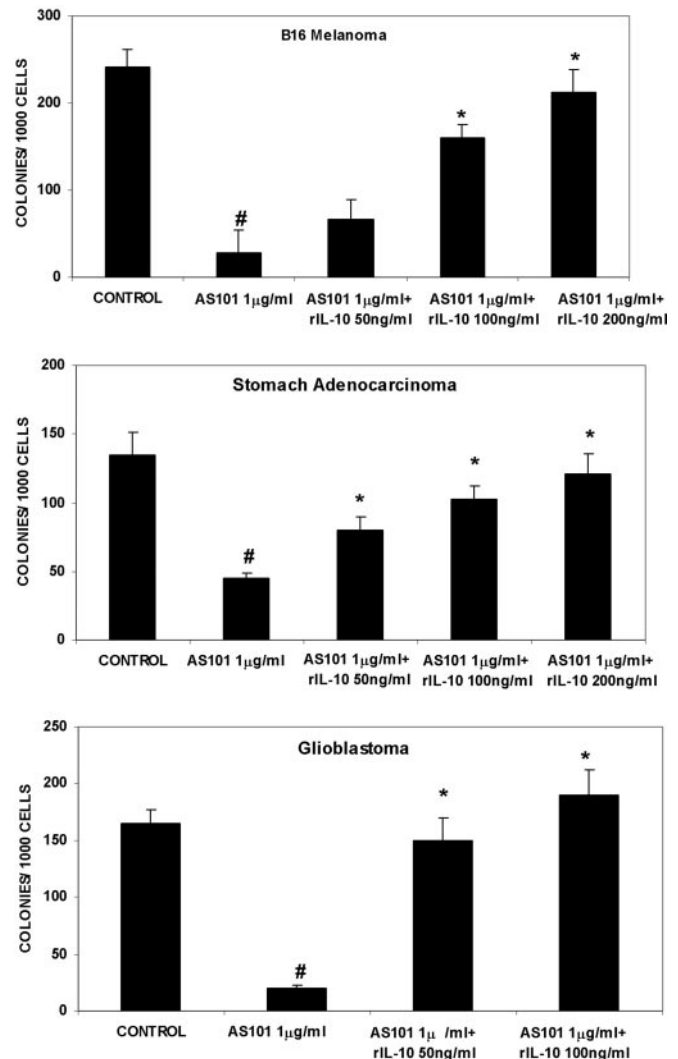


Fig. 5. IL-10 abrogates the inhibitory effect of AS101 on tumor cell proliferation *in vitro*. Tumor cells were cultured in the presence of AS101 at 1 μg/ml and various concentrations of IL-10. Clonogenicity of tumor cells was assessed. #, $P < 0.01$ decrease versus control; *, $P < 0.01$ increase versus AS101. The results represent means from three different experiments; bars, SE.

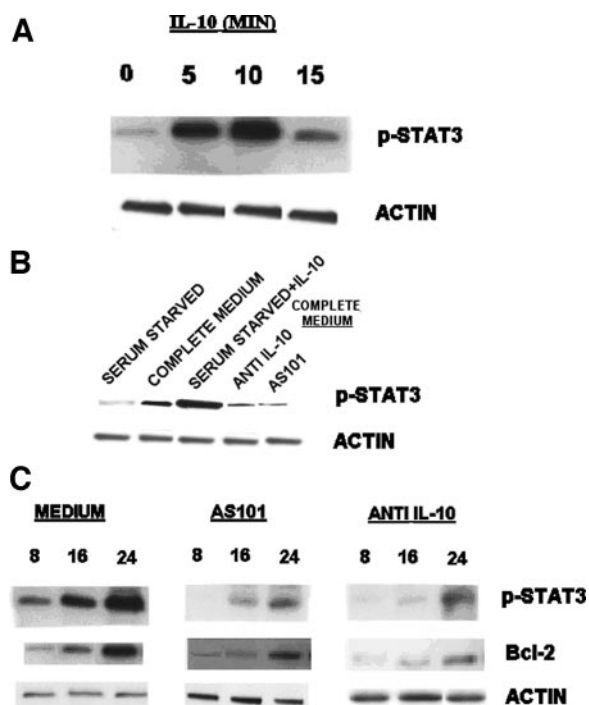


Fig. 6. Role of IL-10 and its inhibition by AS101 on the expression of pStat3 and Bcl-2 in B16 melanoma cells. 24-h serum-starved cells were incubated with 100 ng/ml of recombinant murine IL-10 for various time points. Expression of pStat3 was assessed by WB analysis (A). Cells were either serum starved or cultured in the presence of complete medium containing serum. IL-10 (100 ng/ml) was added to serum-starved cells for 10 min. AS101 at 1 μ g/ml or anti-IL-10 neutralizing antibodies (10 μ g/ml) were added to cells in complete medium for 24 h. Expression of pStat3 was assessed by WB analysis (B). Cells were cultured in complete medium for various time points in the presence of either AS101 (1 μ g/ml) or anti-IL-10 neutralizing antibodies (10 μ g/ml). Expression of pStat3 and Bcl-2 was assessed by WB analysis (C). The results show one representative experiment of three performed.

incubated serum-starved B16 cells in the presence of IL-10 for 10 min and compared expression of phosphorylated Stat3 in these cells to that in cells cultured in complete medium. In both conditions, pStat3 was more prominently expressed as compared with serum-starved cells. The expression of pStat3 in cells cultured in the presence of IL-10 was even higher than that in cells cultured in complete medium (Fig. 6B). Supplementation of either AS101 or anti-IL-10 neutralizing antibodies to B16 cells cultured in complete medium almost completely abrogated expression of pStat3 (Fig. 6B).

Dephosphorylation of Stat3 by AS101 or by Neutralization of IL-10 Decreases Expression of Bcl-2. Members of the Bcl-2 family of proteins are important regulators of programmed cell death pathways with individual members (*e.g.*, Bcl-2 and Bcl-X_L) that can suppress apoptosis and promote survival (26). Bcl-2 expression was found previously to be attributable to the control of Stat3 (27). We therefore thought to determine in the following experiments if inhibition of pStat3 by AS101 via reduction of IL-10 will affect expression levels of Bcl-2. As can be seen in Fig. 6C, starved B16 melanoma cells cultured in medium + serum expressed increased phosphorylated Stat3 in a time-dependent manner. This protein expression was substantially inhibited by both AS101 and anti-IL-10 antibodies. Increased phosphorylation of Stat3 in control cells was positively correlated with increased levels of Bcl-2. Moreover, inhibition of pStat3 by either AS101 or anti-IL-10 abs was associated with decreased expression of Bcl-2. These data suggest that AS101 may reduce expression of Bcl-2 after inhibition of Stat3 activation via IL-10 inhibition.

AS101 Sensitizes B16 Melanoma Cells to Chemotherapy.

Down-regulation of Bcl-2 has been implicated previously in increased sensitivity to chemotherapy. We therefore wanted to ascertain whether dephosphorylation of Stat3 by AS101, resulting in reduced Bcl-2 expression, will increase sensitivity of B16 cells to conventional chemotherapy. Fig. 7A shows that incubation of B16 cells with AS101 for 24 h significantly increases the sensitivity of tumor cells to paclitaxel, doxorubicin, and 5-fluorouracil. This was reflected by the increase in the percentage of inhibition of clonogenicity from 33% (paclitaxel), 32% (doxorubicin), and 30% (5-fluorouracil) to 65, 55, and 50% (chemotherapy + AS101), respectively ($P < 0.01$). Furthermore, AS101 increased the magnitude of apoptosis exerted by the various chemotherapeutic drugs 2–3-fold, increasing the proportion of apoptotic cells from 15% (paclitaxel), 17% (doxorubicin), and 15% (5-fluorouracil) to 46, 43, and 41% (chemotherapy + AS101), respectively ($P < 0.01$; Fig. 7B).

AS101 Increases Sensitivity of Human GBM to Paclitaxel *in Vitro* and *in Vivo* Via Inhibition of IL-10. Glioblastomas constitute >30% of all brain tumors and are characterized by a high degree of malignancy and invasiveness. Chemotherapeutic agents, including paclitaxel, used in the treatment of GBM with limited success, have been shown to act primarily by inducing apoptosis (28). On the basis of our results showing the ability of AS101 to inhibit proliferation of GBM cells via interference with the IL-10 autocrine loop and the role of IL-10 inhibition in the sensitization of B16 cells to various che-

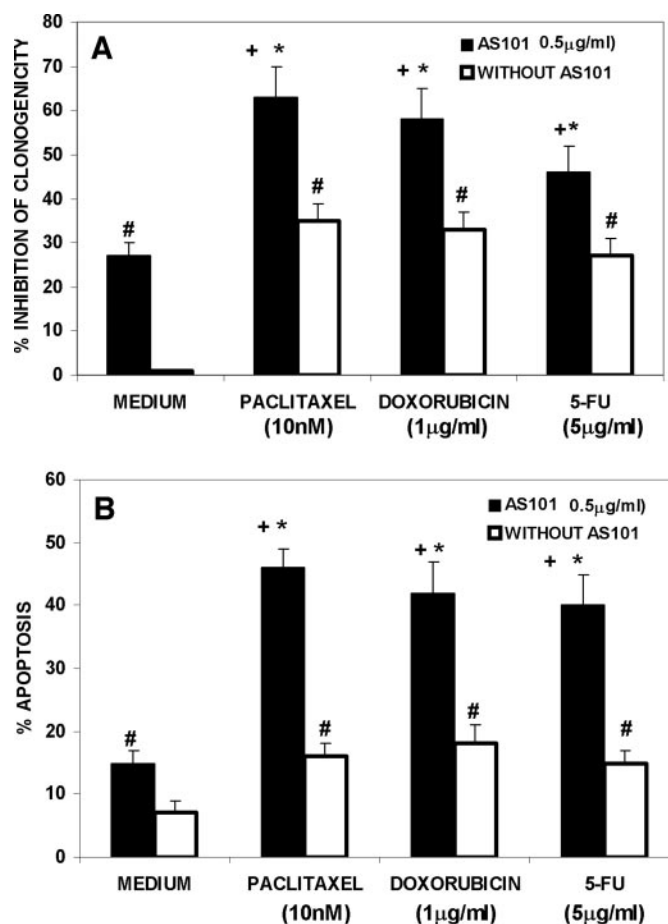


Fig. 7. AS101 sensitizes B16 melanoma cells to chemotherapy. B16 melanoma cells were cultured for 24 h in the presence or absence of AS101 at 0.5 μ g/ml. Various chemotherapeutic drugs were then added. Clonogenicity (A) and apoptosis (B) were then assessed. #, $P < 0.01$ increase versus medium without AS101; *, $P < 0.01$ increase versus medium with AS101; +, $P < 0.01$ increase versus chemotherapy without AS101. The results represent means from three different experiments; bars, SE.

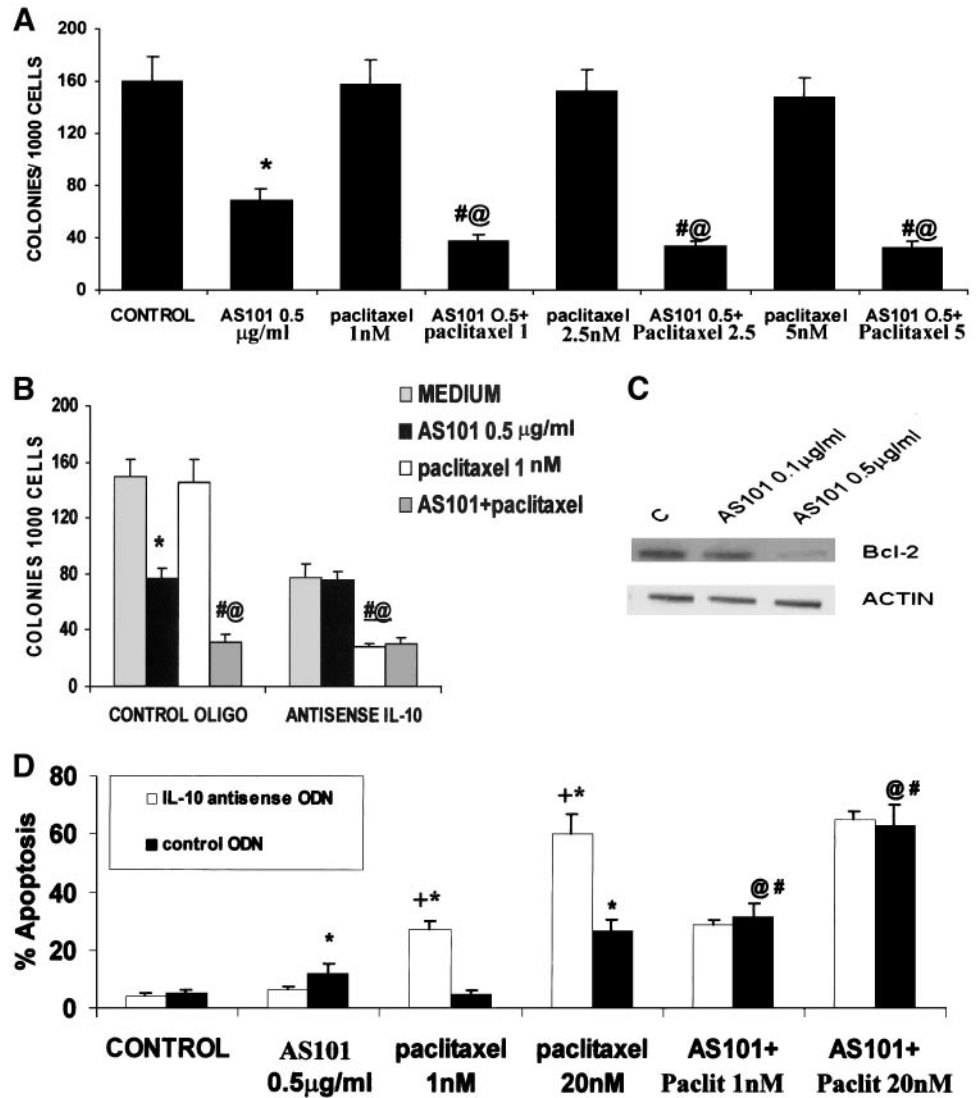


Fig. 8. AS101 sensitizes GBM tumor cells to paclitaxel in an IL-10-dependent manner. GBM cells were cultured for 24 h in the presence or absence of AS101 at 0.5 $\mu\text{g/ml}$. Paclitaxel at various concentrations was then added. *A*, clonogenicity was assessed. *B*, GBM cells were transfected with either IL-10 antisense ODNs or with control ODNs and processed as in *A*. *, $P < 0.01$ decrease versus medium; #, $P < 0.01$ decrease versus AS101; @, $P < 0.01$ decrease versus paclitaxel. *C*, GBM cells were cultured without or with AS101 at 0.5 or 1 $\mu\text{g/ml}$ for 24 h. Expression of Bcl-2 was assessed by Western blot analysis. *D*, GBM cells were transfected with either IL-10 antisense ODNs or with control ODNs and cultured as in *A*. Apoptosis was assessed. *, $P < 0.01$ increase versus medium; #, $P < 0.01$ increase versus AS101; @, $P < 0.01$ increase versus paclitaxel; +, $P < 0.01$ increase versus control ODN. *A*, *B*, and *D*: The results represent means from three different experiments; bars, SE. The data in *C* represent one of three experiments performed.

motherapeutic drugs, we wanted to determine whether this property of AS101 could enhance the sensitivity of GBM cells to paclitaxel *in vitro* and *in vivo*.

In these experiments, we preincubated primary cultures of glioblastoma with AS101 and added paclitaxel 24 h later at concentrations that do not inhibit GBM proliferation. Fig. 8A shows a synergistic inhibition of clonogenicity at all paclitaxel concentrations tested ($P < 0.01$). Transfection of antisense IL-10 ODNs to GBM cells decreased the number of colonies from 150 ± 12 to 78 ± 9 ($P < 0.01$), suggesting a significant role of IL-10 in GBM cell proliferation (Fig. 8B). AS101, which, as expected, decreased clonogenicity of control cells transfected with mismatched ODNs, did not decrease proliferation of antisense IL-10 ODNs, suggesting that inhibition of GBM proliferation by AS101 is exerted via IL-10 inhibition. Moreover, sensitization of GBM cells to paclitaxel was equally effective when cells were either treated with AS101 or were transfected with IL-10 antisense ODNs (Fig. 8B). These results demonstrate that AS101 sensitizes GBM cells to inhibition of proliferation by paclitaxel via inhibition of IL-10. Fig. 8C shows that AS101 greatly decreased expression of Bcl-2 in a dose-dependent manner. This activity was associated with increased magnitude of apoptosis exerted by AS101 and paclitaxel at either 1 nM (increase from 4.8 ± 1.2 to $31.5 \pm 4.6\%$; $P < 0.01$) or 20 nM (increase from 26.4 ± 4.1 to

63.2 ± 7.3 ; $P < 0.01$; Fig. 8D). The apoptosis in GBM cells induced by paclitaxel was IL-10 dependent, because apoptosis was augmented in GBM cells transfected with IL-10 antisense ODNs. AS101 did not further increase the level of apoptosis in these transfected cells, implying that this agent sensitizes GBM cells to paclitaxel via IL-10 inhibition (Fig. 8D).

The *in vitro* results prompted us to explore the ability of AS101 to sensitize GBM tumors to paclitaxel *in vivo*. For this purpose, we used a SCID mouse xenograft model in which we implanted control GBM cells or cells transfected with IL-10 antisense ODNs and treated mice as described in "Materials and Methods." Fig. 9A shows that GBM tumors became evident relatively late after tumor cell implantation. On day 28, tumor weight was low in all groups, and no significant differences were observed between groups. Nevertheless, on day 46 (a time where mice in some groups started to die) tumor weights of AS101 or paclitaxel-treated mice were significantly decreased ($P < 0.01$). AS101 and paclitaxel exhibited a strong antitumoral synergistic effect, which was abrogated by cotreatment with IL-10. Mice transplanted with GBM cells transfected with IL-10 antisense ODNs (efficiency of transfection presented in Fig. 9B) developed smaller tumors, similar to those in AS101-treated mice transplanted with control GBM cells. Moreover, tumors arising from ASN-ODN-transfected cells were highly responsive to paclitaxel (Fig. 9A).

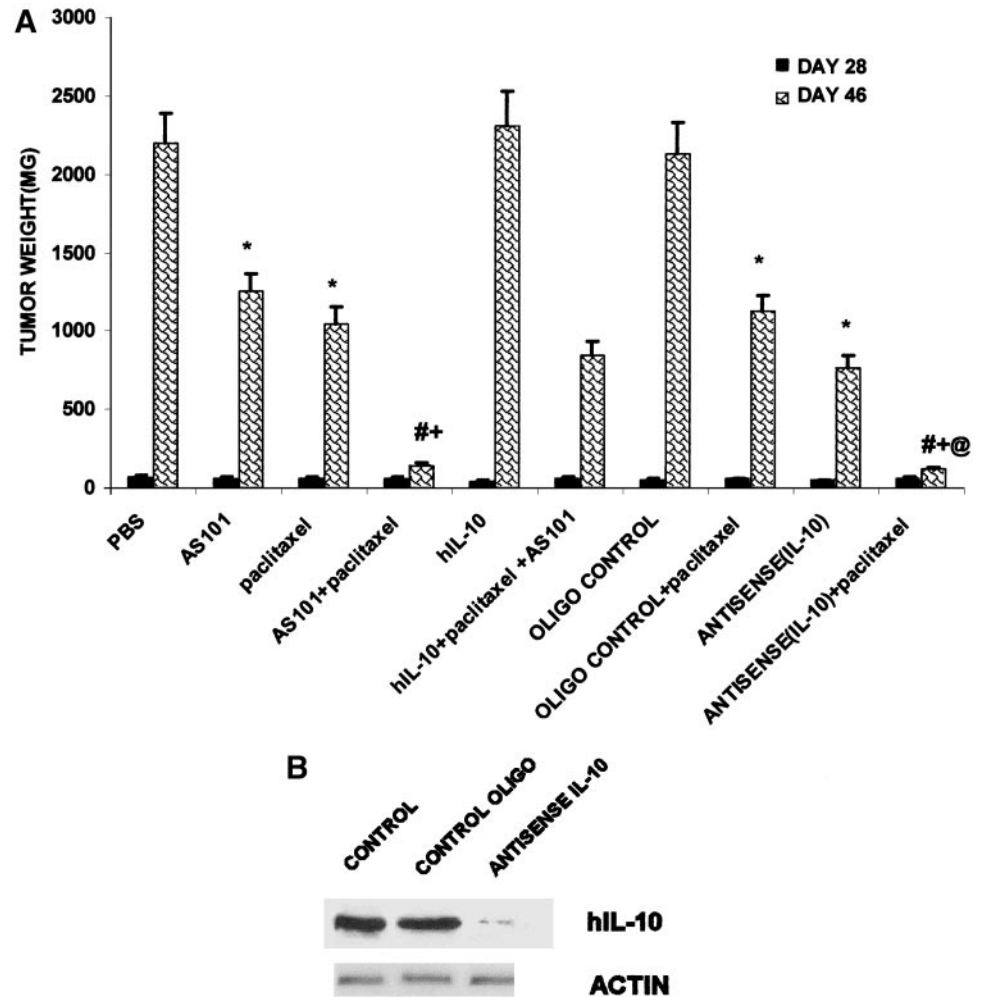


Fig. 9. AS101 sensitizes GBM tumors to paclitaxel via inhibition of IL-10, resulting in decreased tumor weights. SCID mice were transplanted with human GBM tumor cells and treated as described in "Materials and Methods." A, tumor weight was estimated on days 28 and 46. Data represent means of tumor weight estimations from a total of 100 mice; bars, SE. *, $P < 0.01$ decrease versus PBS; #, $P < 0.01$ decrease versus AS101; +, $P < 0.01$ decrease versus paclitaxel; @, $P < 0.01$ decrease versus antisense IL-10. B shows the efficiency of IL-10 antisense ODN transfection by Western blot analysis.

We then wanted to evaluate whether production of IL-10 by GBM tumors is correlated with specific treatment outcome. As can be seen in Fig. 10A, human IL-10 levels, reflecting IL-10 secreted by tumor cells, were very low in mice treated with AS101 + paclitaxel, or in mice transplanted with GBM cells transfected with AS-ODN, or in mice transplanted with transfected cells and treated with paclitaxel. Although these low levels of IL-10 in the first and third groups may reflect the small tumor weights, the IL-10 levels of the second group probably reflect the inability of tumor cells, regardless of tumor size, to produce IL-10. Supplementation of IL-10 to the treatment protocol (groups 10 and 11) increased serum IL-10 levels regardless of tumor size, partly because of injected IL-10 remaining in serum. Fig. 10B reflects the ability of tumors to produce IL-10 regardless of their size; the figure shows the amount of hIL-10 expressed in 25 μ g of tumor protein. All treatment protocols involving either AS101 or AS-ODN-transfected cells reduced IL-10 protein expression, whereas others, similar to paclitaxel alone, although reducing tumor weight, did not decrease IL-10 expression by the remaining tumor cells.

We then wanted to determine whether the decrease in tumor weight is reflected by a parallel increase in survival. Fig. 11A shows that both paclitaxel and AS101 significantly increased survival of GBM tumor-bearing mice, with paclitaxel being more effective than AS101. Daily treatment with rIL-10 decreased survival, resulting in all mice dead at day 56 compared with day 68 for control PBS-treated mice. Cotreatment with both drugs resulted in 45% survival at day 80 ($P < 0.01$). Addition of IL-10 to this cotreatment protocol totally abrogated the therapeutic effect of AS101 and paclitaxel; such mice had poorer

survival than those treated with AS101 or paclitaxel alone (Fig. 11A). Fig. 11B shows that when mice received injections of GBM cells transfected with IL-10 antisense ODNs, their survival increased beyond that of mice injected with control ODN-transfected cells ($P < 0.05$). Moreover, paclitaxel treatment of mice transfected with IL-10 antisense ODNs resulted in a significantly increased survival that was similar to that of mice injected with control cells and treated with AS101 and paclitaxel ($P < 0.01$; Fig. 11B). These results suggest that targeting IL-10 alone either by AS101 or by injection of cells transfected with AS-ODN, although resulting in increased survival, does not exert dramatic effects on GBM tumor-bearing mice. However, IL-10 inhibition increases the response of GBM tumor-bearing mice to paclitaxel. The sensitivity of the tumor to paclitaxel is enhanced both *in vitro* and *in vivo* by inhibition of IL-10. Nearly all of the effects of AS101 on the tumor appear to be mediated through IL-10 inhibition,

DISCUSSION

In the present study, we present evidence that three different tumor cell lines, two of which are human tumor primary cultures, constitutively secrete IL-10 in an autocrine/paracrine manner. We show that this cytokine is essential for their proliferation, which is significantly decreased by AS101 via inhibition of IL-10. We further show that this inhibition results in dephosphorylation of Stat3, which is constitutively expressed by tumor cells, an event that is followed by decreased expression of the antiapoptotic protein Bcl-2. Moreover, these activ-

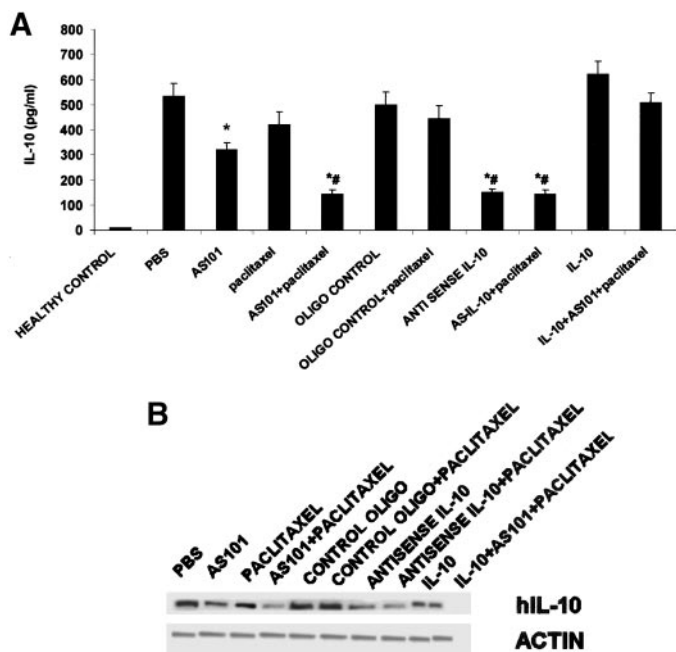


Fig. 10. IL-10 is produced in GBM tumor-bearing mice by the tumors. SCID mice were transplanted with human GBM tumor cells and treated as described in "Materials and Methods." On day 42, three mice from each group were sacrificed, sera were collected for the evaluation of IL-10 content (A), and tumors were dissected for preparation of cell lysates for the evaluation of human IL-10 protein expression. Data in A represent means \pm SE from three different mice/group. Data in B represent one of three dissected tumors/group.

ities of AS101 are associated with sensitization of tumor cells to various chemotherapeutic drugs, resulting in their increased apoptosis. More importantly, we show that AS101 sensitizes a human aggressive GBM tumor to paclitaxel both *in vitro* and *in vivo*, and it does so primarily through IL-10 inhibition. As a result, increased survival and a pronounced decrease in tumor weight are obtained in mice cotreated with AS101 and paclitaxel.

The autocrine stimulation of cell proliferation by IL-10 has been observed previously in human melanoma (3) and myeloma (29). In addition, IL-10 has been shown previously to be expressed in human gliomas (6), and high circulating IL-10 levels in gastric cancer and Hodgkin's disease patients were positively correlated with poor disease-free survival (30, 31). Although the B16 melanoma cell line has not been described previously to secrete IL-10, IL-10-transfected cells grew faster than control cells both *in vitro* and *in vivo* (12). We show for the first time that both primary cultures of human GBM and gastric adenocarcinoma, as well as the murine B16 melanoma cell line, produce IL-10, which stimulates their proliferation in an auto-crine manner. IL-10-induced tumor cell proliferation is probably mediated, at least in part, by activation of the latent transcription factor Stat3, which is essential for all known IL-10-mediated immune responses (32). We show that B16 melanoma cells constitutively express activated Stat3, the phosphorylation of which is greatly decreased after neutralization of IL-10. A number of studies suggest that aberrant Stat signaling, in particular Stat3, participates in the development and progression of human cancers by either preventing apoptosis, inducing cell proliferation, or both (14). Inhibition of constitutive Stat3 activation in diverse tumor cell lines by blocking tyrosine kinase signaling has been associated with growth suppression and induction of cell death (33). Many Stat3 target genes are known, including those encoding the antiapoptotic proteins Bcl-xL, Mcl-1, and Bcl-2, the proliferation-associated proteins cyclin D1 and Myc, and the proangiogenic factor vascular endothelial growth factor (34). In addition, Stat3 cooperates with c-Jun to repress expression of Fas

(35), presumably interfering with cancer cell apoptosis. AS101 has been shown previously to up-regulate Fas/Apo-1 expression on B16 melanoma cells, an effect that was associated with the *in vivo* antitumoral activity of the compound (36). Thus, it is tempting to suggest that inhibition of Stat3 activity by AS101 via inhibition of IL-10 may derepress Fas expression on B16 melanoma cells, contributing to their increased apoptosis. Moreover, the importance of this activity of AS101 is highlighted by the findings that low Fas expression is associated with resistance to therapy, metastatic capacity, and poor prognosis.

An important outcome of inhibition of tumor cell-derived IL-10 by AS101 is the sensitization of the tumor cells to chemotherapy. In B16 melanoma cells, we illustrate the importance of endogenous tumor-derived IL-10 in the constitutive signaling of Stat3 transcription factor; the inhibition of its signaling by either AS101 or anti-IL-10 not only correlated with decreased Stat3 phosphorylation but also with the reduced expression of Bcl-2. Using luciferase assays, it has been shown previously that Stat3 increased the Bcl-2 promoter activity in a pre-B-cell line (27). Moreover, IL-10 is a known promoter of Bcl-2 expression (37, 38). Bcl-2 is the best characterized member of the Bcl-2 family of proteins, having been shown to protect tumor cells from apoptotic stimuli, including drug cytotoxicity (39). Inhibition of Stat3 phosphorylation and Bcl-2 expression in B16 melanoma cells via AS101-mediated inhibition of IL-10 is associated with increased sensitivity to a variety of cytotoxic drugs. These results are consistent with those of Alas and Bonavida (38) and Stassi *et al.* (40), who have described a similar phenomenon in non-Hodgkin lymphoma B cells and thyroid cancer cells *in vitro*. We have shown previously an increased sensitivity of B16 melanoma tumors to paclitaxel *in vivo* and in mice cotreated with AS101 and paclitaxel (23). However, in

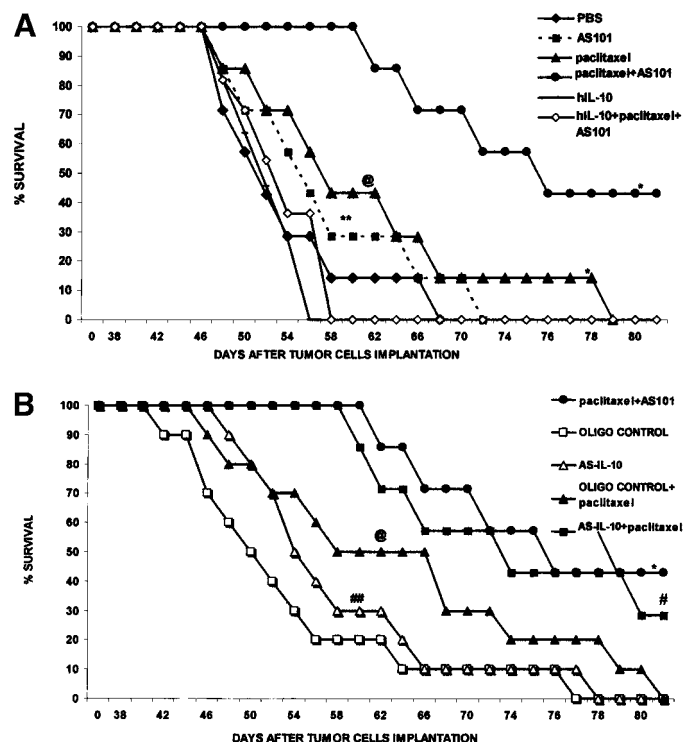


Fig. 11. AS101 sensitizes GBM tumors to paclitaxel via inhibition of IL-10, resulting in increased survival. SCID mice were transplanted with human GBM tumor cells and treated as described in "Materials and Methods." Survival experiments in A and B were monitored for 80 days. The survival data represent a total of 102 mice. *, $P < 0.01$ increase versus AS101 or paclitaxel; **, $P < 0.05$ increase versus PBS; @, $P < 0.01$ increase versus PBS or oligo control; #, $P < 0.01$ increase versus antisense IL-10; ##, $P < 0.05$ increase versus oligo control.

that study, the increased activity was attributed to a transient accumulation of the cells in G₁ after treatment with AS101, driving them to increased G₂-M arrest after treatment with paclitaxel. Because blocking of Stat3 signaling in B16 melanoma has been reported to induce cell cycle arrest (41), it is tempting to speculate that inhibition of IL-10 by AS101, deactivating Stat3 *in vivo*, may result in G₁ arrest, concomitantly with reducing the levels of Bcl-2.

The role of IL-10 in the proliferation of the three types of malignant cells examined in this study, the ability of AS101 to reduce their proliferation via inhibition of IL-10, and particularly the ability of AS101 to sensitize B16 melanoma cells to chemotherapy, prompted us to evaluate the role of IL-10 and its inhibition by AS101 in the sensitivity of the malignant GBM to chemotherapy, both *in vitro* and *in vivo*.

Glioma, and in particular its most malignant form, GBM, is a highly vascularized brain tumor that displays rapid and invasive growth restricted to the central nervous system. Glioblastomas are consistently resistant to standard treatment approaches. On the basis of promising studies on the effects of paclitaxel on cultured glioma cells (42), several clinical studies of paclitaxel for human malignant glioma patients have been conducted (42, 43), resulting in disappointing outcomes. The data in our study show that AS101 sensitizes GBM cells to low concentrations of paclitaxel in an IL-10-dependent manner, and this effect is correlated with decreased expression of Bcl-2 in AS101-treated cells.

Increasing evidence suggests a role for the antiapoptotic Bcl-2 family members in glioblastoma carcinogenesis, treatment resistance, and disease progression (44). Bcl-xL is expressed in all glioblastomas and is up-regulated in patients demonstrating recurrent or progressive disease. Therefore, the importance of the ability of AS101 to reduce Bcl-2 expression in melanoma and GBM cells is highlighted by the fact that antisense-mediated inhibition of Bcl-2 has been reported to chemosensitize both human malignant glioma (45) and human melanoma (46).

Although our *in vitro* data emphasize the role of autocrine IL-10 in the proliferation of human GBM, we show in our *in vivo* model, that inhibition of IL-10 by either AS101 or IL-10 antisense ODNs, although increasing the percentage of survival and decreasing tumor weight, does not affect these parameters dramatically. This can be explained by the presence of other tumor growth factors affecting the progression of the tumor. However, the most prominent role of IL-10 inhibition, as illustrated in this study, is sensitization of the tumor, *in vivo*, to Taxol. Systemic administration of AS101 has been shown recently to attenuate 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced loss of tyrosine hydroxylase-immunoreactive cell bodies in the substantia nigra of mice (manuscript in preparation),⁸ suggesting that AS101 may transverse the blood-brain barrier and preserve dopaminergic neurons in this Parkinson disease-like syndrome in mice. Thus, by entering into the central nervous system, AS101 might sensitize GBM tumors to the very low concentrations of Taxol penetrating into the brain at doses that are tolerated when given systemically.

Interfering with IL-10 by AS101 could possibly result in the restoration of antitumor immune responses, because this cytokine has been implicated in its down-regulation. IL-10 renders tumor cells totally insensitive to CTL lysis (47). We have shown previously that treatment of tumor-bearing mice and advanced cancer patients with AS101 leads to a clear dominance in TH1 responses associated with the antitumoral effects of AS101 (22).

The identification of Stat3 as the most well-characterized down-

stream target of IL-10, coupled with the widely reported aberrant activation of Stat3 signaling in malignant progression of human cancers, implicates the IL-10-Stat3 signaling pathway as a novel molecular target for therapeutic intervention in cancer.

The data provided in this study show that the nontoxic immunomodulator AS101, currently being used in clinical studies, inhibits the IL-10-Stat3 autocrine signaling loop, sensitizing tumor cells to chemotherapy *in vitro* and *in vivo*. These findings, coupled with the reported angiogenic activity of IL-10 and Stat3 and the ability of IL-10 to provide a means to escape immune surveillance, suggest that AS101 in conjunction with chemotherapy may provide an alternative for the treatment of IL-10-dependent tumors. Moreover, given the urgent need for new therapeutic strategies against gliomas in general and glioblastomas in particular, the findings that an agent such as AS101, which sensitizes GBM tumors to very low concentrations of paclitaxel, imply that this agent may be effective in the treatment of IL-10-dependent gliomas, even when combined with the limiting concentrations of paclitaxel that enter the central nervous system.

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