Growth Inhibition of Human Melanoma Tumor Xenografts in Athymic Nude Mice by Swainsonine

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ABSTRACT

Swainsonine, an inhibitor of α-mannosidases, has been shown to block experimental metastasis of B16F10 melanoma and MDAY-D2 lymphoid tumor cells in syngeneic mice. In this report we demonstrate that swainsonine also reduces the growth rate of human melanoma cells in vitro and in vivo. Graded doses of swainsonine were administered either orally or via implanted Alzet miniosmotic pumps to athymic nude mice bearing subcutaneously implanted human MeWo melanoma cells. Swainsonine at 10 µg/ml in the drinking water or 0.5 mg/kg/day administered by miniosmotic pump reduced the growth rate of the MeWo tumors by approximately 50% and inhibited the expression of complex-type oligosaccharides in tumors and host intestine by only 10–20%. Swainsonine doses of 4 mg/kg/day reduced expression of complex-type oligosaccharides by 85% in vitro but afforded no additional inhibitory effect. A glycosylation mutant of MeWo called 3S5 has a defect in the synthesis of complex-type asparagine-linked oligosaccharides resulting in incomplete processing similar to that observed in swainsonine-treated MeWo tumor cells. Swainsonine did not inhibit the proliferation of 3S5 cells in vitro nor the growth of 3S5 tumors in nude mice. The results suggest that expression of highly branched complex-type oligosaccharides commonly associated with the malignant phenotype may provide the tumor cells with a growth advantage.

INTRODUCTION

Neoplastic transformation in both murine and human tumor cells is often accompanied by increased —GalNAcβ1-6Manα1-6Manβ1— branching of the trimannosyl core in complex-type asparagine-linked oligosaccharides as shown below (1-5).

SAα2-3Galβ1-4GlcNAcβ1-6Manα1-6Manβ1— branching of the trimannosyl core in complex-type asparagine-linked oligosaccharides as shown below (1-5).

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Studies on chemically transformed hamster embryo cells have shown that some transformed lines retained glycopeptides similar in size to those of their normal cell counterparts (6). However, serial passage of these transformed cells in animals is accompanied by a shift to larger N-linked structures, suggesting that enhanced expression of β1-6-branched oligosaccharides may be a characteristic of more malignant tumor cell subpopulations that are inadvertently selected during prolonged tumor cell growth in vivo (7).

Recent studies in several murine tumor models also support the idea that tumor cells expressing highly branched asparagine-linked oligosaccharides have a selective growth advantage at the primary site of tumor growth and that these cells also selectively metastasize (8). Transfection of the nonmetastatic mammary tumor cells called SP1 with pSV2neo induced metastatic phenotype in 10–40% of the isolates. The metastatic sublines in a mixed population of transfected SP1 cells could be identified by their unique neo integration site. These cells: (a) showed elevated levels of β1-6-branched oligosaccharides; (b) dominated the tumor cell population at the site of injection, and (c) consistently populated the metastatic nodules (8, 9).

Loss or premature truncation of β1-6-branched complex-type oligosaccharides due to mutations affecting the processing pathway results in loss of metastatic potential and a growth disadvantage at the site of tumor cell injection (9-11). The class 1 mutants of MDAY-D2 produce truncated asparagine-linked oligosaccharides lacking galactose and sialic acid, due to an apparent deficiency in Golgi UDP-Gal transporter activity (12). Consequently, asparagine-linked oligosaccharides lack the normal SA-Gal-GlcNAc antennae commonly found in wild-type structures and the cells produce truncated structures as shown below.

Tumorigenicity is not compromised by the class 1 mutation as the number of tumor cells required to produce 50% tumor incidence was the same for both mutant and wild-type cells (i.e., 10-100 cells injected s.c.) (9). The class 1 mutant cells produced a palpable tumor composed of class 1 mutant tumor cells over the course of 30 days. Between 30 and 60 days, the mutant tumor cells were progressively overgrown at the site of injection by class 1 host cell hybrid cells (11). The recessive class 1 mutation was suppressed in the hybrids and as such the tumor cell regained both wild-type oligosaccharide structures and malignant potential (10). These results suggest that reexpression of the wild-type asparagine-linked oligosaccharide structures in the hybrid tumor cells is associated with more rapid growth and a selective growth advantage of the cells in the solid tumor.

If expression of branched complex-type oligosaccharides is associated with a growth advantage in vivo, then drugs such as swainsonine, which inhibit oligosaccharide processing, might be expected to induce a less malignant phenotype similar to that of the class 1 glycosylation mutant. Swainsonine has been shown to inhibit Golgi α-mannosidase II, and therefore complex-type oligosaccharides are replaced with hybrid-type oligosaccharides as shown below (13).

Swainsonine treatment of B16F10 melanoma and MDAY- D2 tumor cells has been shown to reduce their organ colonization potential when the cells are injected into the circulation.
of syngeneic mice (14, 15). The alkaloid has also been shown to inhibit the growth of v-ras-transformed 3T3 fibroblasts in soft agar (16).

In this report, we show that swainsonine administered to athymic nude mice bearing human MeWo melanoma tumors inhibits solid tumor growth. The MeWo cell line expresses the more branched complex-type asparagine-linked oligosaccharides which are characteristic of malignant cells (18). In contrast 3S5, a glycosylation mutant of MeWo, has truncated complex-type oligosaccharides identical in phenotype to the class 1 mutants of MDAY-D2. 3S5 cells proliferated at a reduced rate and were not further inhibited by swainsonine in vivo or in vitro. The results suggest that the antiproliferative action of the alkaloid is related to inhibition of asparagine-linked oligosaccharide processing in the tumor cells.

MATERIALS AND METHODS

Cell Lines. The growth and metastatic behavior of MeWo in athymic nude mice, a human melanoma cell line, has been documented (17). The 3S5 line was selected as a spontaneous wheat germ agglutinin-resistant mutant of MeWo and the cells showed the same phenotypic defect in asparagine-linked oligosaccharide biosynthesis as the class 1 mutants of MDAY-D2 (i.e., glycoconjugates deficient in sialic acid and galactose due to a defect in Golgi UDP-Gal transport) (18).

Tumor Cell Growth Rates. Cell lines were routinely propagated in RPMI supplemented with 5% FCS in a humidified, 5% CO2 atmosphere. To measure growth rates, MeWo and 3S5 tumor cells were plated at 2 x 10^6 cells/ml and serially diluted to 625 cells/ml in serum-free medium, consisting of Iscove's modified Dulbecco's medium, supplemented with 5 µg/ml human transferrin, 1 µg/ml bovine serum albumin, and 1.2 µM ethanolamine. Duplicate wells were counted on the following 4 days and linear regression of the data was used to calculate the doubling times. Cells grown in the presence of swainsonine were cultured in the presence of 1 µg/ml of drug for 48 h prior to setting up the growth screen, and swainsonine was included in the media for the duration of the experiment.

Human Tumor Growth in Nude Mice. BALB/c nude mice were supplied with drinking water containing swainsonine 1 day before receiving an s.c. injection of 10^6 MeWo or 3S5 tumor cells and then maintained on swainsonine-supplemented water for the duration of the experiment. The mice used approximately 5 ml of water/day. Alternatively, miniosmotic pumps with a 2-week supply of swainsonine or water were implanted s.c. into the mice 1 day before injecting tumor cells into the opposite flank. The pumps held 200 µl, delivered 0.5 µl/h, and were replaced 3 times to allow measurement of tumor growth over a 6-week period. Chemically synthesized swainsonine was obtained from Toronto Research Chemicals. Purity was determined by proton nuclear magnetic resonance spectrometry. The alkaloid is chemically stable at room temperature and has previously been administered to rodents over a period of weeks with no overt toxicity (19).

Minimum and maximum tumor diameters were measured twice weekly and used to calculate tumor size index (minimum diameter x maximum diameter). The rates of solid tumo growth were obtained by linear regression of the tumor size index measurements. Tumor size index measurements approximated a linear function with respect to time. Significant differences between tumor growth rates were determined using analysis of variance and the F distribution.

SDS-PAGE and Western Blotting. Tumor cells were lysed in 10 mM Tris, pH 7.5-0.15 mM NaCl-0.5% Triton X-100-2 mM phenylmethylsulfonyl fluoride at a fractional % aprotinin on ice. Mouse intestinal tissue was homogenized in approximately 10 volumes of the same buffer using a Polytron. After centrifugation at 20,000 x g for 15 min, the supernatants were stored at -20°C. Protein concentrations were determined using the BCA reagent (Pierce) and 60 µg of each sample were separated by SDS-PAGE and electrotransferred onto nitrocellulose sheets. The blots were incubated with 0.1 µg/ml of L-PHA in PBS/0.1% BSA for 1 h, washed 3 times in PBS/0.1% BSA and incubated for 1 h with a 1/1000 dilution of rabbit anti-L-PHA antibody. The IgG was then washed 3 times in PBS/0.1% BSA and incubated for 1 h in alkaline phosphatase-coupled goat anti-rabbit antibody diluted 1/3000 in PBS/0.1% BSA. Blots were washed 4 times in PBS/0.1% BSA, then once in PBS/0.05% Tween 20 followed by 1 wash in PBS finally developed according to manufacturer's instructions (Bio-Rad).

Immunoblots to detect LAMP-1 glycoproteins in mouse intestinal homogenates were done using a rabbit polyclonal anti-LAMP-1 antibody raised against purified LAMP-1 (20), followed by a goat anti-rabbit IgG conjugated with alkaline phosphatase and development as described above.

RESULTS

In Vitro Growth of MeWo and 3S5 Cells. In tissue culture, the glycosylation mutant 3S5 grew more slowly than the parental MeWo cell line in the presence or absence of 5% FCS as shown in Fig. 1. The growth rates of both cell lines were sensitive to initial seeding density of the cultures. At low cell densities in medium with 5% FCS, MeWo cells had a doubling time of 50 h as compared to 56 h in the presence of swainsonine. In contrast, the slower doubling time of 3S5 cells in 5% FCS was not lengthened further by swainsonine. A similar disparity in the doubling times of MeWo and 3S5 cells was observed in serum-free medium (Fig. 1B). In the absence of serum, density-dependent growth of swainsonine-treated MeWo cells was very similar to that of 3S5 either with or without swainsonine. This indicates that, under conditions of limiting growth factors, the density-dependent growth profile of swainsonine-treated wild-type cells approximates that of the glycosylation mutant 3S5. Swainsonine appeared to exert a cytostatic rather than cytotoxic effect on the cells since the plating efficiency of the MeWo and 3S5 cells was not reduced by the drug (data not shown). Similar effects of swainsonine on cell growth and viability have been observed for MDAY-D2 lymphoma and HT29m colon carcinoma cell lines (15, 21).

Inhibition of MeWo Melanoma Growth by Swainsonine. The growth rates of subcutaneous 3S5 tumors in nude mice was reduced by 50-65% compared to that of MeWo tumors (Fig. 2, Table 1). The administration of swainsonine at 10 µg/ml in
were injected s.c. into groups of 5 mice, and the tumor size index was determined as described in “Material and Methods.” The mice received 0 (D), 0 (*), 1 (A), 6.0, and 3.0 mm²/day, respectively. The growth of 3S5 tumors (•) in untreated mice was 2.4 mm²/day. The growth of 3S5 tumors and MeWo in mice treated with 10 μg/ml of swainsonine was significantly reduced compared to MeWo in untreated mice, P < 0.01.

The drinking water of nude mice bearing MeWo tumors reduced tumor growth rates similarly, by 54%. Tumor-bearing mice treated with lesser doses of swainsonine in their drinking water did not show significant reductions in tumor growth rates (Fig. 2). Although the latency of 3S5 tumors was longer than MeWo, this parameter of MeWo growth was not affected by swainsonine treatment.

Although oral administration of swainsonine appeared to be an effective route for inhibiting solid tumor growth, it is obviously difficult to regulate and monitor the amount of drug consumed by the mice. Therefore, Alzet miniosmotic pumps containing different concentrations of swainsonine were implanted into nude mice. The pumps were installed 1 day before injecting tumor cells and replaced at 2-week intervals to maintain a constant level of swainsonine in the animals over a period of 6 weeks. The tumor growth rates are summarized in Table 1 and show that 0.5 mg/kg/day or greater produced near optimal growth inhibition of solid MeWo tumors. With the dosages of swainsonine used in this experiment, inhibition of tumor growth did not appear to be dose dependent. However, swainsonine did not significantly reduce the already slower growth rate of 3S5 tumors, which is consistent with the in vitro results shown in Fig. 1. Although the calculated values for growth rates varied between experiments, the differences between groups was consistent (Table 1). The incidence of tumor takes was reduced to 80% in the groups receiving 2 and 4 mg/kg/day of swainsonine. Additional experiments with escalating doses of both swainsonine and inoculated tumor cells are required to determine whether the drug also reduces tumorigenicity.

Effect of Swainsonine Treatment on Oligosaccharide Processing in Vivo. The plant lectin L-PHA requires galactose and the β1-6-linked antenna in tri- and tetraantennary oligosaccharides for high affinity binding (22). Thus, L-PHA can be used to detect these structures in malignant cell lines and monitor their loss following growth of the cells in the presence of swainsonine. Table 1 and Fig. 3 show that inhibition of oligosaccharide processing in the tumors and intestines of animals receiving 0.5 mg/kg/day of swainsonine was reduced by approximately 10–20%. This minimal effect on oligosaccharide processing was also observed in mice given 10 μg/ml of swainsonine orally (data not shown) suggesting that only partial inhibition of oligosaccharide processing is required for optimal inhibition of tumor growth. However, almost complete inhibition of oligosaccharide processing in the MeWo tumors was achieved in mice given 4 mg/kg/day of swainsonine (Fig. 3). The identity and origin of the L-PHA-reactive species with a molecular weight of 70,000 which persisted in the tumors from swainsonine-treated animals is unknown.

As shown in Fig. 3, dose-dependent inhibition of oligosaccharide processing on an individual glycoprotein, LAMP-1, could be monitored by its reduction in apparent molecular weight on SDS-PAGE. The molecular weight of murine LAMP-1 ranges between 100,000 and 160,000 depending on the tissue source. The deduced amino acid sequence contains 20 consensus sequences for asparagine-linked glycosylation (23, 24) and the majority of these sites appear to be substituted with O-linked and N-linked sugars. LAMP-1 ranges between 100,000 and 160,000 depending on the tissue source. The deduced amino acid sequence contains 20 consensus sequences for asparagine-linked glycosylation (23, 24) and the majority of these sites appear to be substituted with O-linked and N-linked sugars. The deduced amino acid sequence contains 20 consensus sequences for asparagine-linked glycosylation (23, 24) and the majority of these sites appear to be substituted with O-linked and N-linked sugars. The identity and origin of the L-PHA-reactive complex-type oligosaccharides in malignant tumor cells and in the normal mouse intestine. Size and size heterogeneity of LAMP-1 is greatest in the mouse intestine and this appears to be due to branching and extension of the

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<th>Swainsonine (mg/kg/day)</th>
<th>Tumor incidence</th>
<th>Latency (days)</th>
<th>Growth rate (mm²/day)</th>
<th>P</th>
<th>Reduction in tumor growth rate (%)</th>
<th>Relative completion of processing (%)</th>
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<td>21</td>
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<td>&lt;0.01</td>
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³ Densitometric scanning of L-PHA-stained glycoproteins with molecular weights of 80,000 to 200,000 migrating in SDS-PAGE as shown in Fig. 3 were used to calculate the mean percentage of completion of oligosaccharide processing relative to MeWo.

² Mean ± SD.

¹ Not done.
antennae with polylactosamine sequences (repeating units of Galβ1–4GlcNAcβ1–3). As expected, the conversion of the complex-type oligosaccharide structures in intestinal LAMP-1 to hybrid-type structures by swainsonine treatment reduced the size and size heterogeneity of the glycoprotein with a similar dose response as that observed for loss of L-PHA reactivity (Fig. 3).

DISCUSSION

The observation that class 1 glycosylation mutants of the murine lymphoma MDAY-D2 and the human melanoma MeWo produce tumors, which grow at substantially reduced rates compared to the wild-type cells, leads to the hypothesis that complex-type oligosaccharides enhance tumor cell growth (10, 11, 18). In this report, we have shown that swainsonine treatment of mice bearing human MeWo tumors reduces tumor growth by 50–60% which is comparable to the growth rate of the glycosylation mutant 3S5. However, the alkaloid did not inhibit the growth of 3S5 tumors, presumably because the cells have a mutation which produces a phenotype similar to that of the swainsonine-treated cells. Complex-type asparagine-linked oligosaccharides have by definition 2 or more antennae, and inhibition of their initiation on the Manα1–6Man side of the trimannosyl core by swainsonine treatment reduces the number of sialylated lactosamine antennae per structure by at least 50%. In the glycosylation mutant 3S5, the antennae appear to be initiated in the usual manner by GlcNAc substitution of the trimannosyl core; however, they are not extended with galactose, polylactosamine, and terminal sialic acid residues. Therefore, the structural defect in mutant cells and that in swainsonine-treated MeWo cells have in common a lack of sialylactosamine antennae on the Manα1–6Man side of the trimannosyl core (see structures in "Introduction").

Inhibition of oligosaccharide processing in MDAY-D2 (15) and MDCK cell lines (25) by swainsonine has previously been shown to be incomplete with approximately 10–20% of the complex-type oligosaccharides persisting based on structural analysis of cellular glycopeptides. Although swainsonine is a competitive inhibitor of β-mannosidases II with a low $K_i$ (i.e., approximately 1 μM) (23), complete inhibition of processing may not be achieved in vivo for several reasons. The drug concentrations in the Golgi or accessibility to β-mannosidase II may be less than optimal in vivo. Secondly, some complex-type oligosaccharides may be produced through the alternate processing pathway which circumvents several enzymatic steps in the major pathway including β-mannosidase II (26). Fortunately, the present results suggest that partial inhibition of oligosaccharide processing may be sufficient to inhibit human tumor cell growth in vivo. Low doses of swainsonine (i.e., 0.5 mg/kg/day) which produced only partial inhibition of oligosaccharide processing in MeWo tumors and in mouse intestine (i.e., 10–20%) showed near optimal inhibition of tumor growth in vivo. Similar results have been obtained with the HT29m colorectal carcinoma in vitro; that is, concentrations of swainsonine which only partially inhibit oligosaccharide processing (i.e., 10–50 ng/ml) produce near optimal inhibition of HT29m human colorectal carcinoma cell proliferation in tissue culture (21). This is consistent with the observation that, although the class 3 glycosylation mutants of MDAY-D2 cells are leaky and have 20–40% of the residual GlcNAc-transferase V activity (i.e., the enzyme responsible for initiation of the β1–6-linked antenna) (9), the mutants show reduced malignant potential in vivo and a reduced growth rate in serum-free medium compared to MDAY-D2 cells.4

In serum-free medium the growth rates of MeWo and 3S5 cells were found to be dependent on the initial seeding density of the cultures, suggesting that an autocrine growth factor stimulates their growth. MeWo cells appeared to be less density dependent than either 3S5 or swainsonine-treated MeWo cells. The slower in vitro growth rate of MeWo cells in the presence of swainsonine was comparable to that of 3S5 cells. Similar changes in density-dependent growth were also observed for MDAY-D2 cells treated with either swainsonine or castanospermine and for class 1 and 3 glycosylation mutants of MDAY-D2.4 It is therefore tempting to speculate that mutations or inhibitors which block asparagine-linked oligosaccharide proc-

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essing somehow interfere with the function of autocrine systems utilized by these cells. In the subcutis of the nude mouse, both tumor- and host-derived growth factors may be limiting, producing a restricted growth factor environment analogous to that of serum-free medium in tissue culture.

Altered asparagine-linked oligosaccharide processing can affect the structural conformation, intracellular location, half-life, and biological activity of glycoproteins (27, 28). The combination and magnitude of these effects appears to differ for each glycoprotein. Since all cellular glycoproteins normally expressing complex-type oligosaccharides are affected by swainsonine treatment and by the glycosylation mutations such as that of 35S, it is likely that their effects on cell proliferation are pleiotropic. These considerations also apply to developmentally regulated and cancer-associated changes in the levels of Golgi enzymes involved in complex-type oligosaccharide processing. Developmentally regulated expression of glycosyltransferases and other proteins involved in oligosaccharide processing may provide a means of modulating environmental or intracellular signals regulating cell proliferation. Consistent with this idea, proliferating tissues such as intestinal and lymphoid cells in the mouse show high expression of β1-6-branched oligosaccharides, while brain and liver have little of these structures (29).

Swainsonine has also been shown to inhibit the growth of human HT29 colon carcinoma tumor xenografts in nude mice (21). Similar to the present results with MeWo, swainsonine also reduced HT29m tumor growth by 50%. Combining systemic HuINF-α2 with swainsonine treatment reduced tumor growth by 78–92%. Swainsonine also showed antiproliferative activity in vitro which was additive with that of human α2 interferon on HT29 colon carcinoma, SN12 renal carcinoma, and A375 melanoma cells.

Swainsonine appears to have several antitumor effects in murine tumor models. Pretreatment of B16F10 melanoma cells with swainsonine has been shown to reduce both short-term lung retention (2–8 h) and the frequency of lung tumors (3 weeks) in an organ colonization assay (16, 30). Inhibition of lung colonization was enhanced by prolonged treatment of the host as well as pretreating the injected tumor cells, suggesting that swainsonine may reduce tumor cell adhesion and/or invasion in the lung as well as subsequent growth of the tumor nodules (16). In this regard, swainsonine has recently been shown to inhibit tumor cell invasion of human amnion basement membranes in vitro (31). Tumor cell secretion of metalloproteases was not inhibited by the alkaloid, but the metastatic tumor cells were more adhesive on amnion basement membrane and extracellular matrix. In addition to effects at the level of the tumor cell, the administration of swainsonine to tumor-bearing mice has been shown to enhance natural and humoral immune responses (32, 33).

To summarize, swainsonine-induced loss of complex-type oligosaccharides in malignant cells not only inhibits metastasis but also reduces tumor cell proliferation in limiting growth conditions both in vivo and in vitro. These observations suggest that swainsonine or other drugs which block branching of asparagine-linked oligosaccharides may be useful in the treatment of cancer patients, either alone or in combination with other agents.

ACKNOWLEDGMENTS

The authors would like to thank Frances Hogue and Lynda Woodcock for their secretarial assistance.

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