Inhibition of Primary and Metastatic Tumor Growth in Mice by Cancer-associated Galactosyltransferase Acceptor

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ABSTRACT

The antitumor activity of a glycopeptide purified from human malignant effusion, termed cancer-associated galactosyltransferase acceptor (CAGA), was assessed in BALB/c mice bearing primary and metastatic tumors. Initial studies with the fast-growing KA31 and slow-growing KB521 Kirsten sarcoma-transformed mouse fibroblast cell lines confirmed their tumorigenicity and metastatic potential. Inoculation of 1 x 10^6 KA31 cells s.c. resulted in palpable tumor formation in recipient animals within 14 days and death within 42 days from primary tumor growth (mean survival, 26 days; total survival, 0%). Inoculation of the slower-growing KB521 resulted in tumor formation in 85% of recipients, and tumor-bearing animals succumbed within 56 days after primary inoculation (mean survival, 48 days; total survival, 15%). Administration of CAGA by i.p. injection as a single dose or series of five daily doses (each 50 μg) inhibited primary tumor growth by 35 to 68% in animals receiving KA31 cells and by 25 to 70% in animals receiving KB521 cells. CAGA increased mean survival 50% from 26 to 38 days and total survival from 0 to 27% in animals bearing KA31-derived primary tumors. In animals bearing KB521-derived tumors, CAGA increased mean survival from 48 to 90 days and total survival from 15 to 50%. Similarly, CAGA was also found to significantly inhibit formation of pulmonary metastases in animals after excision of primary tumors. CAGA administration reduced death from metastatic deposits by 55 to 66% in animals initially inoculated with the KA31 cell line and by 58 to 90% in animals initially bearing primary tumors derived from the KB521 line. There was a corresponding decrease in the number of metastatic deposits per lung after administration of CAGA. Thus, CAGA appears to have potential antitumor activity against tumors with a range of growth rates and appears to inhibit both primary and metastatic tumor growth.

INTRODUCTION

Although a small number of agents have been described which in part normalize the biological properties of transformed and malignant cells (e.g., retinoic acid, cyclic adenosine 3'5'-monophosphate) (4, 6, 10), few compounds have been found to selectively inhibit the growth of transformed cells. The majority of clinically useful chemotherapeutic agents are effective by virtue of their activity against rapidly dividing cells and are not specific for malignant cells. A number of antitumor proteins have been isolated which appear toxic to malignant cells in vitro, but their toxicities do not appear to be specific or selective (5, 7-9, 11).

In previous studies, we identified a low-molecular-weight glycopeptide in human malignant effusions which can serve as an acceptor substrate for a transformation associated galactosyltransferase (12-16). This glycopeptide, termed CAGA, was found to inhibit the growth of polyoma virus-transformed fibroblasts in tissue culture but not the growth of their nontransformed counterparts (13, 16). More recently, CAGA has been found to be selectively toxic for a variety of other transformed cell lines (12, 14).

Preliminary studies also indicated an antitumor activity of CAGA in vivo against solid tumors produced in hamsters from the polyoma-transformed hamster fibroblast (12, 16). However, in view of its uniform growth rate and inability to metastasize, this tumor model is of limited similarity to human disease. Further, these studies were limited by our incomplete knowledge of the pharmacokinetics of CAGA. Such knowledge could be used to design protocols to assess the range of CAGA antitumor activity. The ability to prepare radiolabeled CAGA has allowed us to approach these issues; in another paper, we reported the results of serum clearance and tissue distribution following administration of 3H-CAGA in 3 different species by a variety of routes. The results of those studies included the demonstration of (a) the equivalence of parenteral routes, (b) prolonged CAGA serum half-life, and (c) basic interspecies similarity of CAGA pharmacokinetic properties. These results were used to design further studies on the in vivo biological activity of CAGA. In this report, we describe the effects of CAGA on both fast- and slow-growing tumors in mice. The effects of CAGA on growth of both primary and metastatic tumor deposits are also examined.

MATERIALS AND METHODS

Cell Culture. KA31 and KB521 cell lines, subclones of Kirsten murine sarcoma virus-transformed BALB/3T3 fibroblasts, were the generous gift of Dr. S. A. Aaronson, National Cancer Institute. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum prior to use as described previously (1). Prior to animal inoculation, cells were harvested after brief trypsinization and resuspended in fresh medium at a concentration of 1 x 10^6 cells/ml.

Primary and Metastatic Tumor Growth. Primary tumor growth was assessed after s.c. inoculation in the left flank of 4- to 6-week-old male BALB/c mice (Charles River Breeding Farms, Wilmington, Mass.). Animals used in this study were maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. Each animal received 1 x 10^6 cells (KA31 or KB521) in 100 μl after pilot experiments demonstrated maximal tumorigenicity with inocula containing >5 x 10^6 cells/animal. Animals were examined daily for development of palpable tumors and were sacrificed if tumor formation occurred (mean survival, 26 days; total survival, 0%). Inoculation of the KA31 cell line in the hind foot pad resulted in palpable tumor formation in recipient animals within 14 days and death within 42 days from primary tumor growth (mean survival, 24 days; total survival, 0%). Inoculation of the KB521 cell line resulted in tumor formation in 85% of recipients, and tumor-bearing animals succumbed within 56 days after primary inoculation (mean survival, 48 days; total survival, 15%). CAGA administration reduced death from metastatic deposits by 55 to 66% in animals initially inoculated with the KA31 cell line and by 58 to 90% in animals initially bearing primary tumors derived from the KB521 line. There was a corresponding decrease in the number of metastatic deposits per lung after administration of CAGA. Thus, CAGA appears to have potential antitumor activity against tumors with a range of growth rates and appears to inhibit both primary and metastatic tumor growth.
tumor growth. Tumor mass and that of the remaining body were determined at the time of sacrifice or death secondary to tumor extension.

Metastatic deposits were studied in animals following surgical excision of the primary lesion. BALB/c mice were inoculated with either KA31 or KB521 cells (1 x 10^6/100 µl) in the right hind leg. After 14 days (mice receiving KA31 fibroblasts) or 30 days (mice receiving KB521 fibroblasts), primary tumors (1.5 to 2.5 cu cm) were removed by amputation and cauterization. Animals dying within 48 hr were believed to represent complications of technique and were not included in further analyses. At varying intervals, animals were sacrificed, and pulmonary metastases were quantitated by the method of Wexler (17).

Assay of CAGA Antitumor Activity. CAGA was purified from human malignant effusion (15), suspended at a final concentration of 0.5 mg/ml, and sterilized by passage through a 0.22-µm filter (Millipore Corp., Bedford, Mass.). The 50% lethal dose in BALB/c mice was determined after i.p. injection of increasing volumes (0.05 to 1.20 ml) of CAGA in non-tumor-bearing mice and was found to be 450 µg (approximately 20 mg/kg), manifested as death within 24 hr; the cause of death was not determined. No mortality (up to 4 weeks) was observed in animals given dosages of 15 mg/kg or less, including those receiving up to 5 multiple doses (at approximately 24-hr intervals). No mortality was observed in control animals receiving equal volumes of buffer without CAGA.

Animals received single or 5 daily i.p. injections of CAGA (100 to 300 µl) beginning on the day of initial tumor cell inoculation or 48 hr after amputation of primary tumors. Mortality, primary tumor growth, and development of pulmonary metastatic foci were determined as described above and compared to those in control animals not receiving CAGA. Determinations of primary tumor size and quantitation of pulmonary metastases were determined blindly by E. A. Carter without knowledge of treatment group.

RESULTS

The antitumor effect of CAGA, a glycoprotein isolated from human malignant effusions, was studied in mice bearing primary or metastatic tumor deposits. Initial efforts were directed at assessing the tumorigenicity of 2 Kirsten murine sarcoma virus-transformed BALB/3T3 cell lines in this laboratory. Development of primary tumors and their natural history were examined following s.c. inoculation of KA31 and KB521 cell lines. As demonstrated in Chart 1A, inoculation of KA31 cells resulted in uniform tumorigenicity within 20 days (mean, 13 days). Primary KA31 tumors enlarged progressively with 100% mortality within 45 days and a mean survival of 26 days (Chart 1B). In contrast, primary tumors derived from KB521 cells had somewhat slower growth properties with only 85% efficiency of tumorigenesis and with more prolonged intervals for mean (27 days) and maximal (31 days) tumor development (Chart 2A). There was a corresponding prolongation of survival in animals receiving KB251 cells (mean, 48 days) when compared to animals inoculated with the KA31 line (cf. Charts 1 and 2). It should be noted that we did not observe any instances of tumor regression in untreated animals receiving either KA31 or KB421 cell lines. Following delineation of the natural history of the primary tumors formed after inoculation of KA31 or KB521 cell lines, the effect of coincident parenteral administration of CAGA was assessed. Animals were given 5 daily doses (150 µg) via i.p. injection beginning the same day as inoculation of either KA31 or KB521 cell lines. The serum level of CAGA determined 24 hr after the last dose by coadministration of ^3H-CAGA was not significantly different than that found after single-dose injection (1535 ± 175 (S.D) µl versus 1125 ± 150 cpm/100 µl). As demonstrated in Chart 1B, CAGA was associated with delayed tumor formation and with an absolute decrease (72 versus 100%) in the rate of tumor formation in mice receiving KA31 cells. There was a corresponding significant increase in both mean survival (36 versus 25 days, p < 0.001) and total survival (28 versus 0%, p < 0.0001) when compared to untreated controls. In additional studies, inhibition of tumor growth was quantitated by determination of tumor size relative to total body weight and mean wet tumor weight. As demonstrated in Chart 3, tumors in animals receiving 5 daily injections of CAGA were significantly smaller than in untreated controls, with a 66% reduction in absolute tumor weight and a 68% reduction in tumor weight relative to total body weight (p < 0.001). Both absolute and relative tumor weights were also reduced after a single i.p. injection of CAGA, but less markedly than after daily administration, suggesting a dose-response relationship.

CAGA was also effective in reducing primary tumor formation and growth after inoculation of KB521 cells (Chart 2). Again, tumor formation was delayed, and absolute rate of tumorigenesis was reduced (61 versus 82%, p < 0.005) with a corresponding increase in survival (mean, >90 versus 48 days; absolute survival, 50 versus 15%) compared to untreated controls. Determination of actual relative tumor weights revealed a significant inhibition of tumor growth; there was a 25% reduction in tumor size after a single dose and a 70% reduction after 5 daily doses of CAGA (Chart 4).

In view of the substantial inhibition of primary tumor growth associated with CAGA administration, the biological activity of CAGA was further assessed by examining the formation and number of hematogenous metastatic foci. Preliminary experiments demonstrated that all animals succumbed to local extensions and expansion of the tumor at the site of primary inoculation before there was evidence of pulmonary metastases. Therefore, the formation of pulmonary metastases was assessed after removal of the primary tumors. Pilot studies demonstrated that...
leg amputation 14 days after KA31 inoculation into the thigh or 30 days after KB521 injection resulted in minimal surgical mortality or local recurrence but was associated with significant production of pulmonary metastatic foci in animals surviving resection. All animals dying within the study period were examined and were found to harbor extensive pulmonary metastases; these were identified after i.t. instillation of India ink by the method of Wexler (17). The presence of metastatic tumor was corroborated by histological examination which demonstrated metastatic sarcoma with foci surrounding large blood vessels. In contrast, animals surviving at the end of the study period failed to show any evidence of metastatic disease when sacrificed, suggesting that the observed mortality did indeed reflect metastatic progression after resection of the primary lesions.

Following adequate resection of primary tumors, 54% of animals initially inoculated with KA31 cells succumbed to metastatic tumor (Chart 5A), while 46% of animals inoculated with KB521 eventually died (Chart 5B). As demonstrated in Chart 5, administration of 150 µg CAGA daily for 5 days after recovery from surgical tumor resection diminished metastatic progression 61 and 68% in KA31 and KB521 recipients, respectively. It should also be noted that, even in animals eventually dying of metastatic tumor growth, recurrence appeared to be delayed and survival prolonged. Mean survival in untreated KA31 recipients eventually died (Chart 5A), while 46% of animals inoculated with KB521 (14 days for KA31, 30 days for KB521), amputation was performed. After recovery (40 hr), animals received either buffer (B) or 5 daily i.p. injections of CAGA (150 µg or 300 µg) (O). Development of pulmonary metastatic disease was assessed by the method of Wexler (17). A: mice initially inoculated with KA31 or KB521 fibroblasts (n = 36). B, mice initially inoculated with KB521 fibroblasts (n = 36). B, tumor line only; O, tumor line + CAGA.

**DISCUSSION**

In another paper, we examined the pharmacokinetic properties of CAGA, a glycopeptide isolated from human malignant effusion. Those studies demonstrate the equivalence of the bioavailability of CAGA when administered by a variety of parenteral routes in mice and other species. We also documented the prolonged serum half-life of this glycopeptide and its relative concentration in tumors and kidney. In the present studies, we utilized these pharmacokinetic data to assess the antitumor activity of CAGA in 2 model systems. Solid primary tumors were produced in BALB/c mice by s.c. inoculation of KA31 and KB521 recipients treated with CAGA compared to untreated controls. Thus, CAGA administration was associated with a decrease in the number of animals forming metastases and in the number of metastases in those animals which did form these deposits.

CAGA-treated group; mean survivals in KB521 recipients developing metastases were 81 and 92 days in untreated and treated groups, respectively. The effect of CAGA on growth of systemic metastases was further examined by quantitating the number of metastatic foci in treated and untreated animals (Chart 6). The percentage of animals developing metastases was diminished 69 and 89% in KA31 and KB521 recipients after CAGA, while the number of discrete metastatic foci found in lung tissue in animals forming metastases was reduced 70 and 81% in KA31 and KB521 recipients treated with CAGA compared to untreated controls. Thus, CAGA administration was associated with a decrease in the number of animals forming metastases and in the number of metastases in those animals which did form these deposits.
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KB521-derived cells. Our results confirm the findings of Yogeeswaran et al. (18), who noted the faster growth and more efficient tumorigenicity of the KA31 versus the KB521 subclone. However, it should be noted that, in the present studies, both KA31 and KB521 produced tumors more rapidly than previously reported. Furthermore, we observed no instances of spontaneous tumor regression. These slight differences may reflect minor differences in in vitro growth conditions of the cells prior to animal inoculation. Nevertheless, our results in untreated animals closely resemble the previously described natural history of these tumors. We found both KA31 and KB521 tumor lines to be capable of systemic, presumably hematogenous, spread and thus to resemble more closely many human visceral tumors in their biological activity than do other solid tumor models (18).

In view of the prolonged serum half-life of CAGA, we expected that daily i.p. injection would suffice to maintain significant serum levels. Our studies demonstrated that CAGA was effective in reducing primary tumor formation in both fast (KA31)- and slow (KB521)-growing murine sarcoma virus-transformed cell lines. In addition, CAGA was associated with a significant inhibition of growth of metastatic tumor foci and an increase in survival after resection of the primary tumors. Inasmuch as CAGA was administered only after tumor resection, it would appear that CAGA was capable of eliminating preexisting micrometastases since there was a significant increase in tumor-free survival after CAGA administration. These observations extend the range of apparent CAGA antitumor activity and suggest that this glycopeptide is active in a number of species, in tumors of varying growth rate, and in models displaying both primary and metastatic tumor growth.

The basis of the antitumor activity of CAGA still remains uncertain. This substance was initially identified as a potential acceptor substrate for galactosyltransferase. However, the relationship between the ability of this glycopeptide to act as a substrate for galactosyltransferase and its ability to selectively inhibit transformed cell growth remains uncertain. It is possible that the inhibition of transformed cell growth in vivo is entirely independent of substrate activity. Further, the basis of the selective effect on transformed cells in contrast to their more normal counterparts remains unclear. Although recent studies have demonstrated highest levels of CAGA binding by transformed cells, some nontransformed counterparts appear to bind enough of the glycopeptide to effect inhibition in nontransformed sensitive cells (14). Therefore, the effect of CAGA on transformed and malignant cells appears to depend on factors extending beyond simple cellular binding. It is especially puzzling that a glycopeptide isolated from malignant effusions of patients with advanced disease should exhibit antineoplastic activity. However, Barford has also described the isolation from ascitic tumor models of small glycopeptides which possess antineoplastic activity (2, 3). It is possible that these substances represent fragments of endogenous tumor agents which disrupt tumor-specific processes.

Determination of the mechanism of the antitumor activity of CAGA may provide some insight into processes necessary for promoting growth of both primary and metastatic tumor foci. Studies are currently in progress to define the structural features of CAGA necessary to effect inhibition of tumor growth. Studies in other animal models will also be needed to fully delineate the extent of the activity of CAGA against both primary and metastatic tumor growth.

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Note Added in Proof

Preliminary studies since acceptance of this paper suggest that CAGA does not possess inhibitory activity in the leukemia L1210 ascitic tumor model.

REFERENCES

D. K. Podolsky et al.


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