Loss of Metastatic and Primary Tumor Factor X Activator Capabilities by Lewis Lung Carcinoma Cells Cultured in Vitamin K-dependent Protein Deficient Serum

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ABSTRACT

A highly metastatic line of Lewis lung tumor cells established in fetal bovine serum (10%) was subcultured into normal rodent (mouse or rat) serum or rodent serum made deficient in functional vitamin K-dependent proteins (barium sulfate adsorption or warfarin treatment of animals). Following injection of cells cultured in normal rodent serum into C57BL/6 mice, Factor X activator activity in the primary tumors increased at a near linear rate per gram tumor and attained 5- to 8-fold higher levels than did cells grown in either of the deficient sera. Secondary lung foci were also visible in all mice of the normal-rodent serum groups within 10 days after injection, and by 21 days extensive tumor growth in the lungs had developed. No secondary lung foci were apparent in any mice of the deficient serum groups throughout 21 days of tumor burden. Cells cultured in nonrodent serum (fetal bovine serum) were less proficient than cells grown in normal mouse serum in developing primary tumor Factor X activator activity and producing secondary tumors. Exposure of cells cultured in barium sulfate-treated mouse serum to normal mouse serum for 3 h and 3 weeks prior to injection partially restored primary tumor Factor X activator and metastatic competence. These data strongly suggest that in Lewis lung tumor cells at least one species selective, plasma/serum vitamin K-dependent protein plays a major role in the regulation of metastatic events and demonstrate that there is a positive correlation between primary tumor Factor X activation activity and metastasis.

INTRODUCTION

The involvement of hemostasis with tumor growth and spread is well established. The influx of plasma with its essential proteins into extravascular space is facilitated by a tumor cell vascular permeability factor (1, 2). Tumor cell procoagulants, such as tissue factor, generate thrombin and consequently fibrin networks which facilitate the proliferation of cancer cells (3), aid in tumor angiogenesis (4, 5), and provide a suitable environment for the continued growth of the tumor mass (4, 6). Fibrin formation and the aggregation of platelets also enhance the attachment of tumor cells to the vascular endothelium (7-10), a prerequisite for extravasation and eventual formation of secondary tumors.

LL3 carcinoma arose spontaneously in C57BL/6 mice and has been widely used as a model to investigate coagulation effects on tumor spread and growth. Numerous compounds that effect various stages of blood coagulation or platelet function have been tested as inhibitors of LL metastasis. The effectiveness of many of these compounds has been variable, however, and in many cases depended on whether an i.v. or solid tumor model was employed (11). Anticoagulation of mice by administration of coumarin vitamin K antagonists such as warfarin or via a vitamin K-deficient diet has been consistently shown to inhibit the metastasis of LL cells to the lungs in both models (12-14). The activity of a cancer procoagulant, present in both primary and secondary LL tumors (15), which activates Factor X is diminished by these treatments (12, 16, 17), suggestive of a role for it in LL cell metastasis (18). Moreover, intrinsic vitamin K-dependent proteins of unknown function have also been detected in LL and other tumor cells, and are altered by warfarin, and are altered by changes in the vitamin K status of the host (19-21). It is probable that the Factor X procoagulant is either an intrinsic vitamin K-dependent protein of cancer cells or requires a vitamin K-dependent protein from the host for the expression of Factor X procoagulant activity.

We recently provided evidence that the Factor X activator (procoagulant) of LL cells in culture consisted of a cellular factor and/or receptor and a species selective, plasma/serum vitamin K-dependent protein (22). LL cells grown in media containing FBS catalyzed the activation of Factor X much less efficiently than did cells exposed to media containing mouse serum. Mouse serum, in which the functional vitamin K-dependent protein content was reduced either by adsorption with barium sulfate or by prior treatment of mice with warfarin, was much less effective than normal mouse serum in supporting Factor X activation.

We report here that highly metastatic LL tumor cells cultured in serum from an incompatible species (FBS) or in compatible rodent serum deficient in vitamin K-dependent proteins exhibit a markedly reduced capacity to catalyze Factor X activation in vivo and to form metastatic lung colonies relative to the same cells cultured in normal rodent serum.

MATERIALS AND METHODS

Male C57BL/6 mice (20-30 g) were either purchased from The Jackson Laboratory (Bar Harbor, ME) or bred here at the Wadsworth Center. LL tumor cells were obtained from the Mason Research Institute, Worcester, MA, and were maintained frozen or by passage in mice every 1-2 weeks. DMEM with and without phenyl red or sodium bicarbonate and FBS were purchased from Sigma (St. Louis, MO). Warfarin was resolved and the sodium salts of racemic warfarin and the S(-) isomer were prepared by the method of West et al. (23). Human Factor X prepared from Cohn fraction III was kindly donated by Dr. William B. Lawson of this institution. It was essentially homogenous by sodium dodeyl sulfate-polyacrylamide gel electrophoresis, contained no detectable Factor X4, and only trace quantities of Factor VII which did not affect the activation of Factor X under the conditions of the experiments. Normal mouse and rat serum were either purchased from Pel-Freeze (Rogers, AK) or obtained from animals within the Wadsworth Center. Vitamin K-dependent protein deficient mouse and rat sera were prepared by stirring 0.1 g of barium sulfate with 1 ml of normal serum for 30 min at 5°C. The solid phase was removed by centrifugation and the procedure repeated. Serum containing nonfunctional vitamin K-dependent proteins was obtained from Wistar rats.
VITAMIN K AND TUMOR METASTASIS

(bred at the Wadsworth Center) administered S(-) warfarin (10 mg/kg) 18 h previously. Protein assays were performed with bicinchoninic acid and copper sulfate solutions purchased from Pierce Chemical Co. (Rockford, IL). Animals were asphyxiated with CO2 prior to surgery and/or blood removal or before the tumors became so large as to cause discomfort.

Establishment of LLM Cell Cultures. Primary cultures of metastatic LL cells were obtained from lung tumors produced by LL cells implanted in the hind leg of a C57BL/6 mouse. The lung tumors were excised and washed twice in PBS and twice in DMEM containing 100 

\( \mu \)g of streptomycin and 100 U of penicillin/ml. Individual tumors were then cut into small fragments (1–3 mm) and three to five fragments placed in FBS-coated tissue culture plates. The fragments were allowed to adhere to the surface of the culture dish by incubation at 37°C under a 5% CO2 atmosphere. DMEM (with phenol red and sodium bicarbonate, pH 7.6)/10% FBS (2 ml) containing 100 

\( \mu \)g of streptomycin and 100 U of penicillin/ml was then added and the cells allowed to grow out from the tumor fragments. The medium was changed daily. After 5 days the tumor fragments were removed and the remaining attached cells were collected from the culture dish by trypsinization, inoculated into 75-cm2 tissue culture flasks and maintained in the same medium. Subsequent isolation of nonadherent cell lines was accomplished by withdrawing the cells which did not attach and propagating them in the same medium. The cells were reseed every fourth day by dilution of 106 cells into fresh medium. Initial in vivo trials with one line of detached cells (LLM4), their attached counterparts, and the LLM13 primary tumor attached cells used in our previous investigations (22) demonstrated that the former were by far the more metastatic; producing higher numbers of secondary lung colonies and affecting 100% of the animals (N = 10) exposed to them. These findings are consistent with those of Young et al. (24, 25) who also observed that rounded, nonadherent LL cells exhibit higher metastatic capability than adherent cells which spread during culture. In subsequent studies, LLM cells were transferred to the appropriate test serum and acclimated for at least 3 weeks before injection into mice. The medium was changed every fourth day.

Animal Studies. LLM cells grown in the appropriate media for 4 days were pelleted by gentle centrifugation, washed and resuspended in phosphate buffered saline. They were counted with a Coulter counter, and diluted in the same media to 106 cells/0.2 ml for injection s.c. into the thighs of mice. Prior to injection, cell viability was determined by Trypan blue exclusion. Since viability was routinely of the order of 90%, no corrections for the number of viable cells injected were made. Warfarin sodium salt was administered to animals in the drinking water as described previously (22).

The isolation of primary tumor cells was accomplished by finely mincing washed tumor sections in PBS with scissors. The heavy particles were allowed to settle briefly and a portion (0.1 ml) from the middle section of the suspension was removed and injected s.c. into the hind limbs of mice.

Assays. The determination of Factor X activator activity in primary tumors was performed essentially as described previously (22) except that sonicates of washed primary tumor cells were incubated for 15 min with Factor X (20 

\( \mu \)g) instead of for 30 min. Tumor tissue used in the assay was sectioned from an area that was free of necrosis. The extent of Factor X activation was determined from the rate of p-nitroaniline cleavage from the chromogenic Factor Xa substrate S-2222 (N-benzoyl-L-glutamyl-glycyl-L-arginine-p-nitroanilide hydrochloride).

The assay of Factor X activation by cultured cells was also performed essentially as described previously (22); the protein content in incubation mixtures containing sonicates of cultured LLM cells was kept constant at 0.15 mg. Factor Xa produced by cultured LLM cells and their primary tumors also shortened the clotting time of Factor VII and X deficient bovine plasma, demonstrating that the cells activate Factor X to a form capable of participating in coagulation. The extinction coefficient used for p-nitroaniline was 8400 mol/liter/cm.

Prior to examination for secondary tumors, the lungs were filled with approximately 2 ml of Bouin’s solution introduced through the trachea.

RESULTS

In vitro, LLM cells in FBS grew slightly more rapidly than cells in either normal rat or mouse serum, which grew slightly faster than cells in barium sulfate-treated mouse or rat serum or the serum from warfarin-treated rats. When injected into animals, however, the primary tumor growth rates from the variously treated cells were indistinguishable (see below).

Changes in the Factor X activator activities of primary tumors derived from LLM cells cultured in 10% FBS, normal mouse serum, or barium sulfate-treated mouse serum as a function of time after tumor cell implantation (s.c. in the thigh) are illustrated in Fig. 1. Primary LLM tumors from cells cultured in normal mouse serum consistently exhibited the highest levels of Factor X activator activity which increased at a near linear rate per gram tumor throughout the duration of the experiment. LLM cells cultured in FBS exhibited a biphasic time course profile: low activity was associated with the first 14 days of tumor growth, but thereafter Factor X activator activity increased at a rate which paralleled that of the tumors produced by cells cultured in normal mouse serum. Activity in tumors of LLM cells cultured in the barium sulfate-treated mouse serum, however, remained very depressed throughout. Compared to the primary tumors of cells cultured in normal mouse serum, the rate of Factor X activation was approximately 7-fold slower. The primary tumor weights of the three treatment groups over the 21 days of the experiment are presented in Table 1. No statistically significant differences (P < 0.05) existed between them, demonstrating that the observed variations in Factor X activation were not due to alterations in the rates of primary

<table>
<thead>
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<th>Day</th>
<th>MS (µg)</th>
<th>FBS (µg)</th>
<th>Ba (µg)</th>
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<tr>
<td>7</td>
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<td>8.0 ± 0.3</td>
<td>8.1 ± 0.2</td>
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</table>

Table 1 Primary tumor weights of variously treated mice

LLM cells grown in normal mouse serum (MS), fetal bovine serum (FBS), or barium sulfate-treated mouse serum MS (Ba) were implanted s.c. in the thighs of C57BL/6 mice. At various times thereafter the tumors were removed surgically and weighed. Values are the mean ± standard deviation from four mice.

Fig. 1. Time course of Factor X activation by primary tumor cells derived from LLM cells cultured in normal mouse serum (○), FBS (■), and barium sulfate-treated mouse serum treated with barium sulfate (△). C57BL/6 mice were injected with 106 cells s.c. in the thigh at Day 0. At various times thereafter, the primary tumors were removed and assayed for Factor X activator activity as described in “Materials and Methods.” Values, means ± standard deviation from four mice.
tumor growth. Moreover, no differences in primary tumor growth rates existed between the variously treated groups in any of the experiments cited below, and hence, these data have been omitted.

A comparison of secondary tumor growth between the three groups at 21 days after tumor implantation is presented in Fig. 2. As early as 7 to 10 days, tumors were detectable without the aid of a microscope in all mice injected with LLM cells grown in normal mouse serum. By Day 14, these tumors were readily visible and by Day 21 they had advanced sufficiently to produce significant distortion of the lung (Fig. 2). LLM cells cultured in FBS also produced metastatic lung lesions in all mice exposed to them, but the appearance of these tumors was delayed relative to those produced by LLM cells grown in normal mouse serum. At Day 14 no secondary tumors were detectable, but at 21 days, secondary tumor growth was visible (Fig. 2). On the basis of size, the secondary tumors produced from LLM cells initially cultured in FBS had advanced only to the stage of the 10- to 14-day tumors of the normal mouse serum group (Table 2). In marked contrast to these two groups, the LLM cells grown in barium sulfate-treated mouse serum produced no visible secondary tumors throughout 21 days of tumor burden. Chronic administration of warfarin to mice injected with LLM tumor cells cultured in normal mouse serum also completely blocked the appearance of secondary tumors during 21 days of tumor burden demonstrating that the LLM cell line retained the same sensitivity to coumarin anticoagulant drugs that is characteristic of LL carcinoma.

Preliminary experiments in vitro with the attached line of LL13 cells used in our previous investigations (22) demonstrated the sera from a number of species including human, equine, dog, and guinea pig were incapable of enhancing the Factor X activator activity of cells grown in FBS, whereas rat serum supported the activity comparably to mouse serum. LLM cells were consequently subcultured from normal mouse to normal rat serum to examine potential species compatibility effects on Factor X activator and metastatic competence in vivo. Cells were also subcultured into barium sulfate-treated rat serum and serum prepared from warfarin-treated rats to confirm that the loss of LLM cell-primary tumor Factor X activator and metastatic capabilities produced by barium sulfate treatment of mouse serum in the previous experiment was not due to adsorption of a component which was not vitamin K-dependent by the barium salt. After 3 weeks in culture, animals were injected with the variously treated cells and the experiment described above repeated.

The time courses of the primary tumor Factor X activation produced by these three groups are presented in Fig. 3. LLM cells cultured in normal rat serum behaved in vivo similarly, but with slightly lower specific activity, to those cultured in normal mouse serum (compare with Fig. 1). LLM cells grown in the serum from warfarin-treated rats contained low levels of Factor X activator activity throughout the experiment, which were indistinguishable from those grown in barium sulfate-treated serum. Data pertaining to secondary lung formation in each group at 14 and 21 days after cell implantation are presented in Table 2; the results obtained with LLM cells cultured in normal mouse serum and FBS are included for the purpose of comparison. Lung tumors were readily apparent at 14 and 21 days in all mice exposed to LLM cells grown in normal rat serum whereas the lungs from animals implanted with LLM cells cultured in either of the deficient sera had no visible secondary tumor growth. These results thus provide compelling evidence that the observed reductions in primary tumor Factor X activator activity and metastatic competence of LLM cells cultured in treated rodent serum were due to the removal of one or more critical vitamin K-dependent proteins.
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LLM cells grown in DMEM/10% fetal bovine serum (FBS), normal mouse serum (MS), normal rat serum (RS), normal rat serum adsorbed with barium sulfate (RS(Ba)), or serum obtained from warfarin treated rats (RS(W)) were implanted s.c. in the thighs of C57BL/6 mice. At 14 and 21 days later, tumor foci in the lungs were counted and measured.

Table 2 Lung foci in variously treated mice

<table>
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<th>Media</th>
<th>Day 14</th>
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</tr>
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<tbody>
<tr>
<td></td>
<td>Number</td>
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<tr>
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</tr>
<tr>
<td>MS</td>
<td>2.0 ± 0.0</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>RS</td>
<td>1.5 ± 0.6</td>
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<tr>
<td>RS (Ba)</td>
<td>0.0</td>
<td>0.0</td>
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<tr>
<td>RS (W)</td>
<td>0.0</td>
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* Only lung foci which had turned a brownish-red color were counted.
^ Values are the mean ± SD of four mice.
^ The size of each lung tumor was determined by measurement of the estimated diameter with a caliper.

To determine whether the removal of vitamin K-dependent proteins from the culture medium produced irreversible effects on LLM metastatic and primary tumor Factor X activator competence, LLM cells acclimated in deficient mouse serum were exposed to normal mouse serum for 3 h or were subcultured in normal mouse serum for 3 weeks prior to implantation into mice. The effects of these treatments on Factor X activator activity in vitro are presented in Fig. 4. LLM cells cultured continuously in barium sulfate-treated mouse serum exhibited approximately 9-fold less activity than did cells grown in normal mouse serum. However, this loss in activity was essentially reversed by exposure to normal mouse serum for 3 h or 3 weeks as the cells regained approximately 70% the Factor X activator activity of the same cells cultured continuously in normal mouse serum.

A comparison of the time-dependent development of primary tumor Factor X activator activities from these groups of LLM cells is presented in Fig. 5. The primary tumors of LLM cells initially cultured in deficient serum and subsequently exposed to normal mouse serum exhibited higher levels of Factor X activator activity than cells grown in deficient serum, but lower levels than cells grown in normal mouse serum. There was, therefore, a positive correlation between the level of Factor X activator activity expressed by LLM cells in vitro and the development of the same activity by the primary tumors produced from them. However, LLM cells cultured in normal mouse serum for 3 weeks did produce slightly higher levels of primary tumor Factor X activator activity than did cells exposed for 3 h. Whether additional time in culture would restore a level of activity comparable to that of cells continuously cultured in normal mouse serum is currently under investigation.

Fig. 3. Time course of Factor X activation by primary tumor cells derived from LLM cells cultured in normal rat serum (C), barium sulfate-treated rat serum (C), and serum obtained from warfarin treated rats (C). C57BL/6 mice received 10^6 cells s.c. in the thigh at Day 0. At various times thereafter, the primary tumors were removed and assayed for Factor X activator activity as described in "Materials and Methods." Values, mean ± standard deviation from four mice.

Fig. 4. Factor X activator activity of LLM cells prior to injection into C57BL/6 mice. Original culture conditions were normal mouse serum [MS(none)] and barium sulfate-treated mouse serum [MS(Ba)]. LLM cells were maintained under these media conditions for 3 weeks. A portion of the latter was then subcultured in normal mouse serum for an additional 3 weeks [MS(Ba)3H]. Another portion of the MS(Ba) cells was removed at the end of the second 3-week period and was exposed to normal mouse serum for 3 h before assay [MS(Ba)3H]. For the assay of Factor X activator activity, approximately equal quantities of the variously treated cells were pelleted by gentle centrifugation, washed with PBS and resuspended in 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid buffered DMEM, pH 7.6, without phenyl red. Following sonication, a portion equivalent to 0.15 mg protein was assayed for Factor X activator activity as described previously (22). Values are the average of duplicate determinations and are expressed as the number of pNAs cleaved from S-2222 per minute per mg protein after a 30-min incubation of the sonicate with Factor X.

Fig. 5. Time course of Factor X activation by primary tumor cells derived from LLM cells cultured in normal mouse serum only (C), barium sulfate-treated mouse serum (C), barium sulfate-treated mouse serum followed by 3-h exposure to normal mouse serum (C) and barium sulfate-treated mouse serum followed by 3-week exposure to normal mouse serum (C). Cells were treated as described in Fig. 4. C57BL/6 mice received 10^6 cells s.c. in the thigh at Day 0. At various times thereafter, the primary tumors were removed and assayed for Factor X activator activity as described in "Materials and Methods." Values, mean ± standard deviation from four mice.

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serum group. The tumors were, however, slightly smaller and fewer in number than those produced by LLM cells continuously exposed to normal mouse serum (data not shown). These data thus demonstrate that the loss of metastatic competence by LLM cells cultured in the absence of vitamin K-dependent proteins is readily reversible and support the concept that the extent of metastasis of LLM cells is related to the level of Factor X activator activity expressed by the primary tumors.

In a related experiment, 21-day primary tumor cells from mice initially implanted with LLM cells grown in normal mouse serum and deficient mouse serum (barium sulfate-treated) were passed again into mice. Although this method is imprecise with respect to the number of cells actually injected, LLM primary tumor cells obtained from the deficient serum group consistently expressed higher levels of Factor X activator activity than in the second group of animals than in the first. Relative to comparably treated LLM cells initially cultured in normal mouse serum, the level of Factor X activation by primary tumor cells passed twice in animals was approximately one-half as great. Secondary tumors were also observed in animals treated with the initially nonmetastatic (barium sulfate-treated) cells after 14 days of tumor burden (data not shown).

**DISCUSSION**

Vitamin K is required for the posttranslational modification of specific N-terminal glutamic acid residues of specifically coded precursor proteins to Gla (26, 27). The formation of this dicarboxylic moiety imparts calcium binding ability to the vitamin K-dependent proteins which, in the case of the coagulation Factors II (prothrombin), VII, IX, and X, is necessary for their activation to enzymatically active forms. Gla residues also bind to insoluble barium salts thus facilitating the selective removal of vitamin K-dependent proteins from serum or plasma. To perform its physiological function, vitamin K must undergo a sequence of metabolic events. Vitamin K hydroquinone is the putative cofactor/substrate for a β-carboxylase, which in the presence of an appropriate protein substrate, molecular oxygen, and carbon dioxide, catalyzes Gla formation. Vitamin K 2,3-epoxide is a product of the reaction which is subsequently reduced back to vitamin K by a vitamin K 2,3-epoxide reductase. Recent evidence suggests that warfarin inhibits vitamin K function at the levels of vitamin K and vitamin K 2,3-epoxide reduction (28, 29). Gla formation is consequently depressed and an increase in the concentration of non- or partially carboxylated precursor coagulation factors results. The extent to which these functionally inactive proteins are retained in the endoplasmic reticulum or are secreted into the plasma is largely species dependent.

There is uncertainty regarding the participation of serum/plasma vitamin K-dependent proteins in the activation of Factor X by LL cells. A number of reports have provided evidence that the LL Factor X activator (procoagulant) differs from tissue factor in that it does not require the coparticipation of Factor VII (12, 17, 18). A procoagulant with such properties has been recently isolated from rabbit V2 carcinoma. It is a thiol protease and differs from tissue factor in its molecular weight and amino acid composition (30). Moreover, polyclonal antibodies against the V2 Factor X cancer procoagulant cross-react with a protein from LL as well as from a number of other warfarin-sensitive murine tumors (16) suggesting that Factor X activation in them occurs by similar if not identical mechanisms. Our previous studies, however, established that one or more serum/plasma vitamin K-dependent protein(s), probably Factor VII, markedly enhances Factor X activation by LL cells in culture (22). Support for the involvement of plasma vitamin K-dependent proteins in tumor dissemination was recently provided by McCulloch and George (31) who concluded that warfarin inhibited the metastasis of rat Mith3 mammary carcinoma cells by affecting the coagulation system of the host and not the tumor cells per se. In the present studies, removal of the vitamin K-dependent proteins from the culture media severely depressed the expression of Factor X activator activity of LLM cells in vitro. The activity was almost completely restored by a brief (3 h) incubation of the cells with normal mouse serum thus reinforcing the concept that LL cells bind a species selective, plasma/serum vitamin K-dependent protein that participates in Factor X activation.

Anticoagulation induced either by warfarin administration or by maintenance on a vitamin K-deficient diet has been repeatedly shown to diminish the metastasis of LL tumors and their ability to catalyze the activation of Factor X in vivo (12, 13, 16, 17). On the basis of these observations, it has been proposed that the capacity of tumors to catalyze the activation of Factor X is positively related to their metastatic competence (18). By changing the vitamin K-dependent protein status of the culture medium, we were able to alter the ability of LLM primary tumors to catalyze Factor X activation and hence determine the consequences of these alterations on metastasis. The advantage of this approach is that secondary stress effects on tumor-bearing mice caused by warfarin treatment or maintenance on a vitamin K-deficient diet are eliminated. Potential warfarin-mediated effects on tumor cell cytotoxicity and induction of host defense systems are also removed by this approach, thus facilitating determination of the mechanism of warfarin action.

The data obtained in mice implanted with LLM cells grown in normal mouse serum suggested that an association existed between high levels of primary tumor Factor X activator activity and the extent of secondary tumor formation and growth. To further establish a possible correlation between these events as well as the involvement of vitamin K-dependent proteins, LLM cells were cultured under conditions which: (a) permitted access to vitamin K-dependent proteins of species other than mouse which did not (FBS) and did (normal rat serum) appreciably support Factor X activator activity in vitro; and (b) permitted access to mouse or rat serum from which the vitamin K-dependent protein content had been depleted either by adsorption of functional, Gla containing proteins with barium sulfate or by rendering them nonfunctional with warfarin. In mice implanted with LLM cells maintained in FBS there was an appreciable delay before primary tumors from these cells attained a rate of Factor X activation that was comparable to that of tumors derived from cells cultured in normal mouse serum. The appearance of secondary tumors in the lungs was also delayed and consequently they were fewer in number and not as large. In contrast, LLM cells cultured in normal rat serum produced rates of primary tumor Factor X activation and secondary tumor growth that very closely approximated those of cells cultured in normal mouse serum. Substitution of the normal rodent serum with vitamin K-dependent protein-deficient serum, however, produced only very low levels of primary tumor Factor X activator activity and no detectable secondary tumor formation. Repletion of LLM cells grown in deficient mouse serum (barium sulfate adsorbed) with vitamin K-dependent proteins from normal mouse serum reversed the inhibition of primary tumor Factor X activator activity and restored the ability to produce secondary tumors. Restoration of primary tumor Factor X activator activity and metastatic competence also occurred if cells from nonmetastatic primary tumors were passed again into mice. In every case, therefore, a positive correlation existed between the development of primary tumors...
Factor X activator activity and secondary lung tumor formation.
Implicit in the data just cited is the fact that LLM cells must have serum vitamin K-dependent protein(s) present at the time of implantation if they are to initially develop high levels of Factor X activator activity and produce viable secondary tumors. Even vitamin K-dependent proteins provided by a species-incompatible donor such as bovine will permit the development of rapid rates of primary tumor Factor X activator activity and secondary tumor formation, but for some unknown reason, the onset of these events is relatively delayed. The biphasic development of primary tumor Factor X activator activity suggests that the cells undergo some type of transformation which enables them to "receive" vitamin K-dependent proteins from the host, but much more detailed studies are required to establish the nature of this phenomenon. Presumably, if mice bearing LLM cells cultured in vitamin K-dependent protein-deficient serum could survive long enough, primary tumor Factor X activator activity and metastasis would eventually increase in much the same way they do in tumors derived from cells cultured in FBS. Evidence for this conclusion is that: (a) there is a slow, but definite increase in the specific activity of Factor X activator activity in the primary tumors of mice implanted with cells cultured in deficient media; (b) the effects of culture in deficient serum are readily reversible; and (c) passage in mice of LLM cells cultured in deficient serum restores primary tumor Factor X activator activity and metastatic capability. An initial complement of vitamin K-dependent proteins does not ensure the development of secondary tumors, however, since warfarin treatment of mice bearing LLM cells cultured in normal mouse serum blocked metastasis. On the basis of these observations, vitamin K-dependent proteins play an essential role in two discrete phases of LLM tumor metastasis. First, species-comparable, serum vitamin K-dependent proteins present in or on LLM cells at the time of implantation rapidly impart to the progeny of the initially administered cells the ability to express high levels of Factor X activator activity and metastatic competence. Second, the development of Factor X activator activity in the rapidly increasing tumor mass can only be achieved when the critical vitamin K-dependent protein(s) of the host is present in adequate supply. Warfarin inhibition of metastasis by initially competent cells presumably occurred by reducing the functional vitamin K-dependent protein content in plasma.

One of the most intriguing, and yet puzzling, findings of the present investigations was that LLM cells cultured in serum deficient in vitamin K-dependent proteins were incapable of expressing significant levels of Factor X activator activity and metastatic tumor formation throughout the entire period of primary tumor growth. That is, the LLM cells behaved in vivo much the same way they would if the mice were subjected to whole-body anticoagulation with warfarin or were maintained on a vitamin K-deficient diet. Since LLM cells cultured in deficient serum can adsorb the required vitamin K-dependent protein(s) from normal mouse serum within a few hours and subsequently in vivo express high rates of primary tumor Factor X activator activity as well as secondary tumor formation, why then don’t primary tumor progeny from cells cultured in deficient serum act in analogous manner in the presence of an X activator activity as well as secondary tumor formation, why then don't primary tumor progeny from cells cultured in deficient serum have a vitamin K-deficient diet.

References

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