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VITAMIN K DEPENDENT FORMATION OF γ-CARBOXYGLUTAMATE RESIDUES IN TUMOR MICROSOMES

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SUMMARY: Vitamin K stimulated the incorporation of ^{14}C into proteins when microsomes from melanoma, mammary gland, mast cell and lymphoma tumors were incubated with $Na_2{}^{14}CO_3$. The ^{14}C label in the [^{14}C] proteins was identified as [^{14}C] γ-carboxyglutamate (Gla), which is formed by carboxylation of glutamic acid residues. Carboxylation in tumor microsomes ranged from 2 to 19% of the carboxylation in normal liver microsomes per mg of microsomal protein. Carboxylation in microsomes was completely blocked by 10 μM Warfarin. SDS-polyacrylamide gel analysis of the melanoma [^{14}C] Gla protein(s) revealed one major peak of ^{14}C with an apparent MW of less than 6,000.

Evidence that anticoagulant treatment and vitamin K deficiency reduce tumor growth and inhibit the spread of tumor cells has recently been reviewed by Zacharski et al. (1). It was proposed that 4-hydroxycoumarins inhibited metastases because they inhibited synthesis of the vitamin K dependent clotting proteins and hence blood clotting. The fibrin clot was thought to protect the tumor cells and aid in their spread. However, present evidence suggests that coumarin anticoagulants and vitamin K deficiency may inhibit metastases by a mechanism independent of inhibition of blood coagulation (2-4).

Oral anticoagulants like the 4-hydroxycoumarins are potent inhibitors of vitamin K dependent carboxylation of glutamate residues in proteins to γ-carboxyglutamate (Gla). Vitamin K dependent synthesis of prothrombin which requires carboxylation of glutamate

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residues has been observed in hepatoma cells in culture (5). This would be expected since liver is the site of the synthesis of prothrombin and the other vitamin K dependent clotting proteins. However, Traverso et al. found that vitamin K dependent carboxylation also occurred in microsomes from renal adenocarcinoma cells in culture (6). Therefore we have examined microsomes from a number of solid tumors to determine if enzymes and substrates required for vitamin K dependent carboxylation are present.

MATERIALS AND METHODS

All tumor cell lines were maintained *in vitro* in RPMI-1640 medium supplemented with 10% fetal calf serum (GIBCO, Grand Island, NY), except for the EL-4 lymphoma, which was maintained by *in vivo* ascitic passage in C57BL/6 mice. Tumor tissue was obtained by subcutaneously inoculating one to 10 million washed cells into the anterior dorsal region of syngeneic mice. The B16-F1 and B16-F10 melanomas and the EL-4 lymphoma were inoculated into syngeneic C57BL/6 mice, while the 66.1A mammary tumor and the P815 mastocytoma cell lines were inoculated into Balb/c and DBA/2 mice, respectively. All mice were obtained from Jackson Laboratories, Bar Harbor, ME.

Mice were sacrified after 1-4 weeks when tumors were large enough to assay. Tumors from 3 mice were pooled, minced and homogenized in 3 times their weight of 250 mM sucrose, 100 mM KCl, 5 mM Mg acetate and 25 mM imidazole, pH 7.2 (SIKM buffer). The homogenate was centrifuged at 15,000 x g for 10 min and the supernatant at 100,000 x g for 60 min. The microsomal pellet was surface washed and resuspended in SIKM buffer. Mouse liver microsomes were prepared in the same manner.

Incubations contained 0.5 ml microsomes (1-10 mg protein (7)), 100 mM $MnCl_2$, 6.5 mM dithioerythritol and 2.5 x 10^7 dpm of $Na_2{}^{14}CO_3$ (specific activity 59 mCi/mmole, Amersham/Searle, Arlington

Heights, IL). The addition of 5 μg of menaquinone-3 (gift from Dr. Peter Hauschka, Children's Hospital, Boston, MA) in 0.01 ml ethanol initiated carboxylation and the sealed mixture was incubated at 25° with constant shaking. After incubation, 0.1 ml of 1M Na_2CO_3 was added and the mixture centrifuged at 100,000 x g for 60 min. The pellet was resuspended in SIKM buffer, solubilized with 0.45% Triton and recentrifuged at 100,000 x g. The incorporation of ^{14}C into the TCA insoluble material of the supernatant was measured as described by Esmon and Suttie (8).

RESULTS

Vitamin K stimulated incorporation of ^{14}C into the TCA-insoluble fraction from 3 to 23-fold in microsomes from the 6 tumor lines tested (Table 1). Liver microsomes from C57BL/6 mice were assayed for comparison. Carboxylation in microsomes from mammary tumors was 19% of that occurring in liver microsomes per mg of protein. The other microsomes had from 1.8 to 9.5% of the activity of liver microsomes. Carboxylation in microsomes from the B16-F10 melanoma was linear for 30 min with no further increase after 60 min (Fig. 1). None of the tumors showed an increase in carboxylation after 1 hr. Carboxylation in microsomes from liver was linear for 1 hr with no increase after this time (data not shown). Therefore incubations were routinely carried out for 1 hr. Warfarin at 10 μM completely blocked carboxylation in microsomes from B16-F10 melanoma and EL4 lymphoma tumors.

Identification of [^{14}C] γ-Carboxyglutamic Acid. To determine whether [^{14}C] γ-carboxyglutamate residues were produced in tumor microsomes, a large-scale carboxylation was carried out with microsomes from B16-F10 melanoma tumors. The [^{14}C] proteins in microsomes were solubilized with Triton and the supernatant was dialyzed and lyophilized. The lyophilized material was hydrolyzed in KOH and the products chromatographed on an anion exchange

Table 1 Vitamin K Dependent Carboxylation in Tumors[a]

Tumor Type	Code	cpm in TCA Precipitate from Incubations: No K	cpm in TCA Precipitate from Incubations: Menaquinone-3 Added	cpm of [^{14}C] Proteins per mg Microsomal Protein (7)
Mammary	66.1A	342	2740	835
Melanoma	B16-F10	128[c]	1960[c]	440 ± 5[c]
Melanoma	B16-F1	111[c]	604[c]	180 ± 12[c]
Mast Cell	P815Y	54	814	138
Mast Cell	P815X-2	90	1250	129
T Cell Lymphoma	EL4	96[b]	296[b]	81[b]
Normal Tissue				
Mouse Liver		335[d]	6650[d]	4440 ± 1210[d]

[a]Incubations were carried out as described in Materials and Methods for 60 min. The results are the averages of duplicate incubations from one preparation of microsomes except as indicated.

[b]Results are the average of duplicate incubations from 2 preparations of microsomes

[c]Results are the average of duplicate incubations from 3 preparations of microsomes ± S.E.M.

[d]Results are the average of duplicate incubations from 5 preparations of microsomes ± S.E.M.

column (Fig. 2). Ninety-three percent of the applied ^{14}C eluted in a volume corresponding to that of authentic Gla (Calbiochem, LaJolla, CA). When an aliquot of fraction 15 (Fig. 2) was heated at 100° in 6N HCl for 17 hr, 57% of the ^{14}C was lost. Under these conditions [^{14}C] Gla would theoretically lose 50% of its radioactivity as $^{14}CO_2$. Another sample treated with HCl at 0° lost no significant ^{14}C. We concluded that the [^{14}C] product of vitamin K-dependent carboxylation in microsomes from the B16-F10 melanoma was [^{14}C] Gla.

SDS Electrophoresis of [^{14}C] Gla Proteins From B16-F10 Microsomes. The [^{14}C] Gla proteins from B16-F10 microsomes were analyzed by SDS polyacrylamide gel electrophoresis (Fig. 3). There was only one major peak which accounted for 84% of the recovered ^{14}C and had an apparent molecular weight of less than 6,000. This peak ran ahead of insulin which was used as a low molecular weight marker.

DISCUSSION

Vitamin K dependent carboxylation of glutamate residues to γ-carboxyglutamate has been observed in microsomes from mammary, melanoma, mast cell and T-cell lymphoma tumors. The mammary tumor had more than twice the carboxylating activity of the other tumors. Carboxylation in tumor microsomes was significant as compared to carboxylation in mouse liver microsomes. Liver microsomes from many species have a high level of carboxylation because they are the site of the synthesis of the vitamin K dependent clotting proteins (9). The [^{14}C] carboxylated product was identified as [^{14}C] Gla in microsomes from the B16-F10 melanoma tumor. It is interesting that the latter melanoma tumor had greater than twice the carboxylating activity of the B16-F1 melanoma since the B16-F10 has a much greater ability to metastasize (10).

SDS-polyacrylamide gel analysis of the [^{14}C] Gla protein from the B16-F10 melanoma tumor gave only one major peak of radioacti-

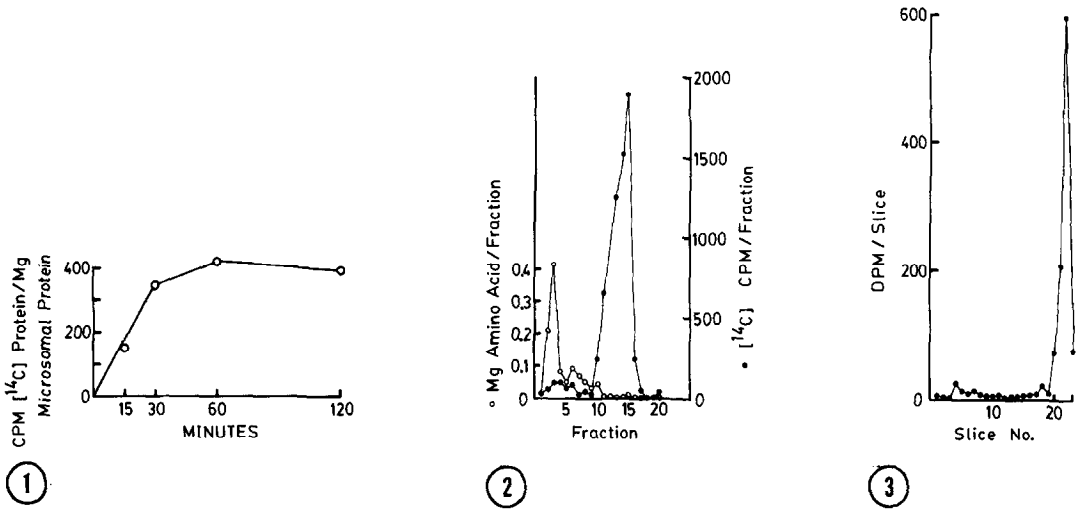

Fig. 1 Kinetics of vitamin K-dependent protein carboxylation in microsomes from B16-F10 melanoma. Incubations were carried out as described in Materials and Methods. The results are the average for duplicate incubations.

Fig. 2 The incubation contained B16-F10 microsomes (40 mg protein) in 6 ml SIKM buffer containing 100 mM $MnCl_2$, 6.5 mM DTE, 3 x 10^8 dpm $Na_2{}^{14}CO_3$ and was initiated with 60 ug of menaquinone-3. After incubation for 1 hr at 25°, 0.6 ml of 1M Na_2CO_3 was added and the incubation mixture was centrifuged at 100,000 x g for 10 min. The pellet was surface-washed with SIKM buffer, resuspended in 3 ml SIKM buffer containing 1 mM benzamidine hydrochloride and 0.45% Triton, and centrifuged at 100,000 x g for 60 min. The supernatant was dialyzed against 2L of 50 mM NH_4HCO_3-1 mM benzamidine hydrochloride with daily changes of buffer until the buffer no longer contained ^{14}C. The dialyzed supernatant was lyophilized and dissolved in 1 ml of 50 mM NH_4HCO_3. The total ^{14}C was 3.1 x 10^4 cpm and the total protein was 3.21 mg (7). An aliquot was hydrolyzed in 2N KOH (13) and chromatographed as described by Gundberg et al. (14). No radioactivity eluted prior to the addition of buffer C. Five ml fractions of buffer C were collected and aliquots were assayed for ^{14}C and for amino acids (15).

Fig. 3 SDS polyacrylamide gel electrophoresis profile of [^{14}C]Gla protein from B16-F10 microsomes. An aliquot of the [^{14}C]protein

vity suggesting that a specific substrate is carboxylated rather than random carboxylation of glutamate residues in many different proteins. The apparent low molecular weight of the [^{14}C] peak raises the possibility that it might be a product of proteolytic cleavage. However, benzamidine, an inhibitor of proteases, was always present during carboxylation and subsequent manipulations.

It would seem reasonable that Gla-proteins serve some function in certain tumors since both the enzyme, vitamin K dependent carboxylase and its substrate(s) are present. For example, Gla-proteins might be required for the spread of cancer cells from the original tumor to other parts of the body. Warfarin, which inhibits metastases of tumors (1), also completely inhibited vitamin K dependent carboxylation in microsomes from the B16-F10 melanoma and EL4 lymphoma. Van Buskirk and Kirsch found that Warfarin caused a 40-50% decrease in cytoplasmic ribosomal RNA from mice hepatomas (11). They later found that purified ribosomes from hepatomas contained Gla residues and postulated that Gla proteins were required for the formation of normal ribosomes and that Warfarin inhibited the formation of these Gla proteins (12). They

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from B16-F10 melanoma microsomes (see legend of Fig. 2) containing 1,500 dpm and 92 µg protein was diluted 1:6 with sample buffer containing 0.125M Tris, pH 8.5, 2% sodium dodecyl sulfate, 10% glycerol, 6M urea, 5% mercaptoethanol, 1 mM EDTA and 0.5% bromphenol blue and heated at 100° for 2 min. Electrophoresis was carried out in a 12 cm 10% SDS polyacrylamide gel (16). The gel was immediately sliced into 5 mm sections which were incubated with 1 ml of 90% NCS (Amersham/Searle) at 45° for 12-16 hr. Nine ml of aqueous scintillation fluid was added and the radioactivity measured. The recovery of ^{14}C from the gel was 77%. Molecular weights were estimated by comparison with electrophoretic migration of known proteins.

also detected Gla residues in ribosomes from human adenocarcinoma and from transformed human fibroblasts.

The melanoma lines with different metastatic abilities developed by Fiedler (10) will give us an opportunity to determine if there is correlation between metastatic ability and the ability to form Gla proteins. Also we can determine if inhibition of Gla protein synthesis by oral anticoagulants is correlated with inhibition of metastases.

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