Autocrine Tumor Growth Regulation and Tumor-associated Hypoglycemia in Murine Melanoma B16 in Vivo

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ABSTRACT

A substance immunochemically cross-reactive with insulin (SICRI) appears in melanoma B16 growing in diabetic and nondiabetic C57BL/6 mice. Progression of tumor size is paralleled by the increase of SICRI levels in the serum of both diabetic and nondiabetic animals; this increase correlates with a decreased concentration of circulating glucose and an elevated concentration of growth hormone in blood. Melanoma B16 grown under serum-free culture conditions secretes SICRI into the medium. Affinity-purified SICRI stimulates glucose uptake by rat epidymyal adipocytes and competes with radiolabeled insulin for binding to these cells. Low concentrations of SICRI enhance growth of cultured melanoma B16 cells, whereas high concentrations of this substance have inhibitory growth effects on these cells. Porcine insulin, human insulin-like growth factors I and II, human growth hormone, platelet-derived growth factor, epidermal growth factor, and fibroblast growth factor have negligible influence on growth of melanoma B16.

INTRODUCTION

Some tumors have been found to produce growth factors whereby they stimulate their own growth and maintain transformed phenotype (1). Recently, we described production and secretion of substances which cross-react with insulin-specific antibodies in several human tumors. The high frequency of association of SICRI with these diseases in humans (2–6) prompted us to look for a suitable experimental model which would enable us to study the production, activity, and structure of SICRI in vivo. In many respects, murine melanoma B16 exhibits SICRI-associated properties analogous to those found in human tumors (2–6) and therefore was chosen for further study.

Growth of melanoma B16 in normal and alloxan-diabetic C57BL/6 mice is accompanied by (a) an increase in levels of SICRI in blood, (b) progressive hypoglycemia in diabetic and nondiabetic animals, and (c) the increase of circulating growth hormone concentrations. Correlations of tumor mass, SICRI concentrations, and glucose in blood as indicators of tumor growth (7). We speculated that SICRI, growth hormone and, possibly, somatomedins were probable candidates for being humoral mediators in an autocrine/endoctrine growth self-incitement mechanism (4, 7).

Here, we report the dynamics of SICRI production and its resultant effects in vivo. Moreover, by in vitro experiments, we provide indirect evidence for mechanistic associations of SICRI concentrations, blood glucose levels, and tumor volume in vivo. Our results show that melanoma B16 could be a suitable model to study autocrine self-regulation in neoplasms and possibly of tumor-associated hypoglycemia and its hypoglycemia-related secondary effects.

MATERIALS AND METHODS

Animals and Tumor. Two and one-half-mo-old male C57BL/6 mice, weighing 22–28 g each, were kept five per cage and fed standard pelleted food and water ad libitum. They were obtained either from Rugjer Boškovic Institute, Zagreb, Yugoslavia, or from The Jackson Laboratory, Bar Harbor, ME.

Melanoma B16, originally obtained from Holt Radium Institute, Manchester, England, has been maintained at the Rugjer Boškovic Institute since 1975 by s.c. inoculations of suspensions containing 2 × 10^6 tumor cells into flanks of C57BL/6 mice. Alternatively, melanoma B16 obtained from the Division of Cancer Treatment Tumor Repository, Frederick Cancer Research Center, Frederick, MD, was used. Three diameters (A, B, and C) of tumor prolate ellipsoids were measured and the tumor volume was calculated as (4πABC/3)2/3.

Diabetes was induced by a single i.v. injection of alloxan, 100 or 120 mg/kg (Merck, Darmstadt, Federal Republic of Germany or Sigma, St. Louis, MO). Diabetes induction was confirmed by the apparent disappearance of circulating insulin (measured by radioimmunoassay) and elevation of glucose concentrations in blood [from 5.5 ± 1.5 (SD)–31.0 ± 5.0 mmol].

Cultivation of Melanoma B16. Melanoma B16-BL6 cells were maintained in a monolayer in RPMI 1640 medium ( Gibco, Grand Island, NY) supplemented with 10% FBS (Gibco) and 20 μM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Cells plated at the density of 1 × 10^5 cells/ml of medium were grown in wells of 24-well plates (Fisher, Rochester, NY) or plastic Petri dishes (35 × 10 mm; Fisher).

In some cases, soft agar was added to the medium in order to establish cell colonies (8, 9). Warm 0.5% agar, 2.5 ml (Gibco) in Eagle’s minimum essential medium ( Gibco) containing 10% FCS or Nu-Serum (Collaborative Research, Lexington, MA) were placed into the 35- × 10-mm Petri dishes and solidified at room temperature. Cells were suspended in 0.85 ml of Eagle’s minimal essential medium with 10% FCS (plating layer) containing 0.3% agar. Triplicate cultures were incubated at 37°C for 5 days in water-saturated air supplemented with 5% CO₂.

Cells were inspected and colonies were counted with 1000 or 2000 using a Nikon inverted phase contrast microscope. Clusters of 30 or more cells were defined as colonies. Cultures were established also from a B16 melanoma grown s.c. in a female C57BL/6 mouse. A cell suspension was prepared by passing tumor tissue through a nylon mesh and three times through a 21-gauge needle and cells were grown in serum-free transferrin-supplemented (5 μg/ml; Sigma) Iscove’s modified Dulbecco’s medium (Sigma). The medium was changed every second day. The first medium change was discarded and the subsequent ones were used as the source of SICRI.

Stimulation of DNA synthesis by growth factors was measured in cells grown in Costar (Cambridge, MA) 96-well clusters (75,000 cells/well) by measuring [methyl-3H]thymidine (specific activity, 2 Ci/mmol; 1 μCi/ml; Research Products International, Mt. Prospect, IL) incorporation. After 24 h incubation of the cells with various growth factors,
Glucose oxidation-Peroxidase kits (Biodynamics, Indianapolis, IN) or by

3. Routinely, 3-4 x 10^5 living cells/ml were used, both for glucose

uptake and insulin/SICRI binding experiments.

4. Cells were incubated in 16-

0.5-ml aliquots of assay

media for 120 min and 250-

pm nylon meshes, centrifuged at 400 x g for 1 min

and washed three times in the albumin-supplemented Krebs-Ringer-

bicarbonate buffer. Finally, adipocytes were washed and suspended in

the assay buffer consisting of 10 mM 4-(2-hydroxyethyl)-1-piperi-

zinesulfonic acid-135 mM NaCl-4.8 mM KCl-2.5 mM CaCl_2-1.7

mM MgSO_4·1.9 mM NaH_2PO_4-bovine plasma albumin, 20 mg/ml-

bacitracin, 0.5 mg/ml (Sigma, St. Louis, MO); 3 mM glucose, pH 7.4,

and incubated at 37°C in 95% O_2/5% CO_2 gas mixture. A small portion

of cells was fixed in 2% OsO_4 in collidine HCl-buffer and counted by

use of a Coulter counter, Model ZB (Coulter Electronics, Hialeah, FL)

(13). Routinely, 3-4 x 10^5 living cells/ml were used, both for glucose

uptake and insulin/SICRI binding experiments.

Glucose uptake by adipocytes was measured by the disappearance of

glucose from the incubation medium (14). Cells were incubated in 16-

x 100-mm Nunc (Roskilde, Denmark) polystyrene test tubes for 2 h at

37°C in the presence or absence of SICRI or insulin. After centrif-

ugation, 20 µl of the assay medium from each tube was mixed with 2

ml of the Glucose oxidase-Peroxidase reagent and the absorbance was

measured at 505 nm following 10 min incubation at 37°C. The amount

declined glucose taken up by adipocytes was calculated as the difference of

glucose concentrations in assay mixtures and in cell-free assay buffer.

SICRI and insulin binding to adipocyte receptors was measured by

competition of SICRI and insulin with 125I-labeled insulin (15). Duplica-

te samples of adipocytes were incubated in 0.5-ml aliquots of assay

buffer containing 125I-labeled insulin, 0.3 ng/ml and various concentra-

tions of unlabeled insulin or SICRI. Non-specific binding was defined

as I_25I-labeled bound to adipocytes in the presence of unlabeled insulin,

100 μg/ml. Incubations were carried out with gentle shaking at 20°C

for 1 h. Duplicate 0.2-ml aliquots of each assay mixture were centri-

fuged through 0.2 ml of dinonylphthalate (BDH, Dorset, England)

in a Beckman Microfuge (Palo Alto, CA). The lower aqueous phase with

unbound insulin was drained through a hole at the bottom of the tube

while radioactivity bound to cells located with upper phase was counted

using a Searle (Des Plaines, IL) gamma counter.

RESULTS

Growth of Melanoma B16 in Normal and Alloxan-Diabetic Mice. We monitored tumor volume and SICRI, glucose, and
growth hormone concentrations in the blood of melanoma B16-

bearing mice as a function of time after tumor inoculation. The

results are shown in Fig. 1. The tumor volume increase (Fig.

1a) was accompanied by an elevation of SICRI concentration in

blood (Fig. 1b), by depression of peripheral glucose levels

(Fig. 1c), and by an increase of circulating growth hormone

concentrations (Fig. 1d). We correlated different data sets from

Fig. 1 by a least squares fitting procedure. The relationship

between SICRI concentration in blood (S) and tumor volume

(V) can be expressed by the empirical equation S = 19 exp(1.6

x 10^-4 V) with r = 0.74, the relationship between S and

circulating glucose concentration (G) by G = 5.3 exp(-1.0

x 10^-3 S) with r = -0.81, and between G and the level of growth

hormone in blood (H) by H = 83 exp(-0.22 G) with r = -0.59

(7). When we performed an experiment similar to that shown

in Fig. 1 in alloxan-diabetic mice, we noted that tumor growth

was similarly accompanied by a decrease of glucose concentra-

tion in blood (Fig. 2), although the growth rate and transplant-

ability were lower compared to normoinsulinemic hosts. No
AUTOCRINE TUMOR GROWTH AND TUMOR-ASSOCIATED HYPOGLYCEMIA

Fig. 1. Melanoma B16 volume (a) and concentrations of circulating SICRI (b), glucose (c), and growth hormone (d) in C57BL/6 mice as a function of time after s.c. inoculation of \(2 \times 10^6\) tumor cells into the flank. Points, mean ± SD (bars) groups of 10-12 animals. \(\mu U\), \(\mu\)units; ml., ml.

Fig. 2. Melanoma B16 volume (a) and blood glucose concentrations (b) in nondiabetic (○) and alloxan-diabetic animals (●) as a function of time after tumor inoculation. Points, mean ± SD (bars) of 10 animals.

reversal of diabetes symptoms was observed in sham-inoculated alloxan-diabetic mice.

Purification of SICRI. Twenty-fold SICRI enrichment can be obtained by polyoxyethylene fractionation. Supernatants obtained by centrifugation of tumor homogenates (usually 25-day-old tumors were used) contained approximately 10 \(\mu\)units SICRI/1 mg of protein. By extrapolating SICRI concentrations in these supernatants to actual tumor volumes, we calculated that the highest SICRI concentration in the tumor was 4700 \(\mu\)units/g. Approximately 60% of measurable SICRI in the homogenate supernatant was precipitated between 10 and 14% POE and for further experiments we used the material precipitated between these two POE concentrations. In none of the tumor extracts was there any growth hormone detected.

In serum-free supernatants of 4- to 5-day-old B16 cultures, SICRI up to 30 milliunits/l could be measured. Specific activity of up to 4.2 milliunits/mg protein was measured in these media after dialysis and concentration. SICRI, affinity purified from these supernatants, had a specific activity of 110 milliunits/mg protein. It was concentrated by use of an Amicon UM-2 membrane to the concentration of 2 units/l and used for tissue culture experiments and chromatography. Thus, the affinity purification step yielded 26-fold purification. The HPLC resolution of POE-precipitated SICRI and porcine insulin on the I-125 column are shown in Fig. 3a. Whereas native SICRI in PBS was eluted in the void volume of the column, in 1 M acetic acid it was coeluted with insulin. This change of elution position was due obviously to dissociation of larger SICRI assemblies. It is interesting that in PBS, native insulin was retarded by the I-125 column.

By use of a calibrated Sepharose 6B column, we found the molecular weight of POE-precipitated SICRI to be approximately 120,000 (Fig. 3, b and inset). The same molecular weight value was obtained by use of culture-derived SICRI and a calibrated Spherogel-TSK 3000 SW HPLC column.

Insulin and SICRI Effects on Rat Adipocytes. Both insulin and SICRI enhanced glucose uptake and they both bound to rat epididymal adipocytes to a similar extent (Fig. 4). The effect of SICRI affinity purified from tumor tissue and from concentrated conditioned culture media was similar. The results of these bioassays were variable and depended on the metabolic status and age of the rats. However, qualitatively they were well

![Fig. 3](image-url)

**Fig. 3.** Molecular sieving chromatography of SICRI and insulin. a, HPLC elution profiles from the I-125 column (7.5 x 250 mm) of 100 \(\mu\)l redissolved melanoma B16 extract, 4800 \(\mu\)units/ml (24 mg protein/ml) [precipitate obtained by precipitation between 10 and 14% POE (○) and of pure porcine insulin (○) in PBS (—•) and 1.0 M acetic acid (—)]. The flow rate was 1 ml/min. The column was calibrated with thyroglobulin \(V_0\), ovalbumin \(V_1\), myoglobin \(V_2\), and vitamin B12 \(V_3\). b, chromatogram of melanoma B16 extract (200 \(\mu\)l of 4800 \(\mu\)units/ml redissolved 10-14% POE precipitate) on a Sepharose 6B 0.9-x 50-cm column developed with PBS containing bovine serum albumin, 0.3 mg/ml at a flow rate of 10 ml/cm²·h⁻¹. The column was calibrated with blue dextran \(V_0\), BSA, IgG, IgM, and \(^{[125]}\)I-labeled bovine insulin \(V_1\), and potassium ferricyanide \(V_2\). Total volume. Inset, plot of log apparent relative molecular mass of human serum albumin, IgG, and SICRI derived from data in b.
In Vitro Stimulation of Melanoma B16 Proliferation by SICRI. In order to assess the effects of SICRI on melanoma B16 cells, we measured in vitro cell proliferation (either as total protein accumulation or [methyl-3H]thymidine incorporation) as a function of SICRI concentration under various conditions.

SICRI effects on cell proliferation were determined in serum-supplemented B16-BL6 cultures, but in serum-free cultures, SICRI almost fully compensated for the growth promoting activities of serum, even at the low concentrations of SICRI (0.5 μunits/ml). However, in the presence of SICRI, 0.5 μunits/ml the absolute proliferation rate was almost the same as the rate in fully supplemented SICRI-free medium (data not shown).

The growth rates of the B16-BL6 cell line as a function of SICRI and insulin concentrations were compared (Fig. 5). The effects of these two substances were strikingly different. While SICRI produced a dose-response effect typical for mitogens (16), porcine insulin did not exert an effect except for a slight inhibition of growth at the highest insulin concentrations, although this insulin is physiologically active in mice in vivo (17).

From the mechanistic point of view, it is imperative to determine whether the growth promotion can be ascribed only to SICRI. Therefore, we compared the effects of affinity-purified SICRI on growth of B16-BL6 cells with analogous effects of the redissolved 10–14% POE precipitate. As can be seen in the inset of Fig. 5, within the limits of experimental accuracy, the results are indistinguishable showing that most of the mitogenic activity in these preparations is associated with SICRI.

SICRI-stimulated [3H]thymidine incorporation was obtained when concentrated and dialyzed (through a molecular weight cutoff = 1000 membrane) tissue culture medium conditioned by melanoma B16 was used (Fig. 6). Here strong stimulation of thymidine incorporation was followed by inhibition of incorporation at higher SICRI concentrations. SICRI affinity purified from these conditioned media exerted similar effects in B16 cell cultures (Fig. 6).

Effects of Growth Hormone, IGF I, IGF II, PDGF, EGF, and FGF on Melanoma B16 Proliferation in Vitro. If growth hormone and/or somatomedins enhance tumor growth, these substances could be indirect SICRI-dependent mediators of tumor growth autostimulation. Therefore, we examined melanoma growth response (measured as appearance of colonies in soft...
growth factors on melanoma cells grown in plastic under identical conditions did not differ significantly from control values showing that among these growth factors only SICRI had a prominent effect.

DISCUSSION

We have recently demonstrated that SICRI concentrations in blood of melanoma B16-bearing mice correlate with the volume of this tumor and that autostimulation contributes to melanoma growth (7). In this report, we show that SICRI acts as the mediator of this response and that it may be capable of affecting glucose transport in the host.

SICRI production and secretion in diabetic mice with B16 melanoma and especially its secretion into serum-free culture media demonstrated that this entity was indeed produced by the melanoma B16. This B16 tumor secretes SICRI into blood, and the circulatory SICRI level was closely correlated with tumor volume. This feature distinguishes SICRI from other tumor-produced growth factors which are predominantly contained in situ (18).

Molecular Size of SICRI. We have been using the term “substances immunohemically cross-reactive with insulin” for tumor-associated insulinoids because at this time we do not know their genetic and structural relationships to “immunoreactive insulin,” the preproinsulin gene products. However, the molecular sieving data reported in this paper shed some light on the gross size of SICRI allowing comparison with insulin.

Under nondenaturing conditions, the relative mean molecular mass of SICRI is 120,000 as in non-Hodgkin’s lymphoma (5). Acid denaturation, however, reduces the molecular weight value of SICRI to that of monomeric insulin suggesting that under physiological conditions SICRI is an aggregate. The precise relationship between tumor-produced SICRI and pancreatic insulin remains to be determined by other methods. However, functional differences between SICRI and insulin indicate that the two entities differ to a certain extent.

SICRI-induced Hypoglycemia and Elevated Growth-Hormone Levels in Blood. Tumor-associated elevation of circulatory SICRI concentrations was accompanied by marked depression of glucose levels both in diabetic and nondiabetic animals. Although we did not have enough purified material to study the effects of i.v.-administered SICRI on circulatory glucose levels, we believe that SICRI-stimulated glucose uptake by rat adipocytes confirmed the relationship between elevated SICRI levels and glucose concentration in blood. If this is true, it would be important to explore whether fluctuations in circulatory SICRI concentrations (6) are related to hypoglycemic episodes in malignant diseases (19) and whether elevated levels of growth hormone in blood (4) are induced by fluctuations of glucose concentrations in circulation (20).

Mitogenic Effects of SICRI. The redissolved precipitate of the tumor extract obtained by POE fractionation (10–14% POE) and the affinity-purified SICRI isolated from this POE preparation exerted similar mitogenic action on B16-BL6 cells in culture. The same pertains to the action of serum-free medium conditioned by the B16 cells and to SICRI affinity purified from this source. These different SICRI preparations exerted maximum effects at somewhat different concentrations. This can be due (a) to possibly different sensitivities (21) of two different melanoma cell lines to SICRI, (b) to differing potencies of intracellular SICRI isolated from tumor tissue and of secreted SICRI, (c) to the presence of other growth-promoting substances in crude POE precipitate and (d) to combinations of the first three factors.

Growth of melanoma B16 cells in culture was stimulated only by SICRI and was not increased by insulin, IGF I, IGF II, growth hormone, EGF, PDGF, and FGF. This exclusive susceptibility to stimulation by SICRI is surprising because it was shown for other systems that the more responsive a cell line in vitro is to one growth factor the more responsive it is to all of them (21).

Melanoma B16 as a Model of Autocrine Stimulation and Tumor-associated Hypoglycemia. Most reports on autostimulation mechanisms in tumors deal with in vitro action of tumor-derived growth factors. Therefore, studies of tumor autostimulation in vivo are of particular interest. For these studies, melanoma B16 appears to be a good model because: (a) it is an easily measurable, fast-growing solid tumor which can be grown in serum-free media for comparative in vitro studies; (b) it produces the growth-promoting SICRI, (and possibly other growth factors) and releases it in blood. As long as more specific assays are not available, SICRI can conveniently be measured by insulin-specific RIA; and (c) melanoma B16 growth and SICRI secretion can be partially manipulated by transplanting the tumor from normoinsulinemic to diabetic hosts. This treatment initially will slow down both tumor proliferation and SICRI secretion (22). Furthermore, study of SICRI-associated hypoglycemia might provide insight into some of the origins of hypoglycemics which often accompany tumors in humans (19).

However, for the purpose of such studies, experimental tumors in their high passages might not be the best objects, because during the first few passages cell populations undergo extensive selection (23). In spite of that, SICRI-secreting tumors might be relatively convenient because by transplanting them into diabetics, a controlled selection pressure can be applied (22). Such systems might be useful for further studies of mechanisms of clonal selection in tumors and tumor-induced derepression of latent genes.

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