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Altered Growth Control by Natural Growth Inhibitors in the Mutant HD33 Substrain of the Ehrlich–Lettré Ascites Mammary Carcinoma

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Abstract—*The mutant substrain HD33 of the Ehrlich-Lettré ascites mammary carcinoma (EAC) was found to be altered in its ability to respond to and to produce natural growth inhibitors. Cells of this substrain did not respond to* (1) *a highly purified growth inhibitor from bovine mammary gland, inhibiting the proliferation of two strains of the original EAC already at concentrations of* 0.5–2.0 ng/ml, (2) *inhibitory activities found in the ascites fluid of these sensitive strains and partially purified. However, from the ascites fluid of the mutant substrain an inhibitory activity was partially purified, which was inhibitory towards this substrain. It also inhibited the cells from an original strain, although less effectively. This new inhibitory activity is similar in its properties to that of the others investigated in that* (1) *its mol. wt is between* 10,000 *and* 50,000 *D,* (2) *it is heat labile and* (3) *its activity can be overcome by insulin, epidermal growth factor and* 2′*-deoxycytidine,* (4) *the dose-response curve levels off at* 35–45% *inhibition. The results demonstrate that the regulation of cell proliferation by endogenous (autocrine) inhibitors can be altered by mutagen treatment.*

INTRODUCTION

INHIBITORY growth regulators are considered to be more or less tissue- or cell-line specific. According to the theory of Bullough and Iverson this is especially true for chalones [1]. Due to the paucity of purified inhibitors, experimental evidence is still scarce and incomplete. To the few examples demonstrating specificities belong: highly purified epidermal chalone [2]; hemopoietic stem cell inhibitors [3–6]; granulocytic chalone preparations [7]; the hemoregulatory pentapeptide [8, 9]; highly purified preparations of a chalone-like factor for the JB-1 ascites tumour [10, 11]; a factor from human serum inhibiting normal and malignant mammary epithelial cells [12] and an inhibitor found in the supernatant of cultures of kidney epithelial cells [13].

We have described the isolation of a factor from bovine mammary gland, inhibiting the resumption of growth of EAC cells *in vitro* [14]. This factor of a mol. wt of about 13,000 D is heat-labile, and its activity is counteracted by such growth factors as insulin, proinsulin, EGF and those present in fetal calf serum and by 2′-deoxyribonucleosides such as 2′-deoxycytidine [15–18]. A similar inhibitory activity had earlier been detected in EAC cells as well as the ascites fluid of EAC bearing mice [16].

We have now found that a mutant HD33 substrain of the EAC was refractory against the purified inhibitor from bovine mammary gland. This prompted us to look for inhibitory activity in the ascites fluid in mice with this special tumour and to compare it with that of two original EAC strains (including the one serving us as test strain for the isolation of the inhibitor from bovine mammary gland). The results show that inhibitor specificity cannot only be found with regard to a given tumour or tissue but can also be induced between different strains of one and the same tumour by mutagen treatment.

MATERIAL AND METHODS

Materials

Medium 199 supplemented with Hanks salts,

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and calf serum (CS) were obtained from the Staatliches Institut für Immunpräparate, G.D.R. Proinsulin free porcine insulin was supplied by VEB Berlin-Chemie, G.D.R. The epidermal growth factor (EGF) was purchased from Serva. α-Amylase (EC 3.2.1.1.) was obtained from Merck. All other chemicals obtained commercially, e.g. 2′-deoxycytidine (CdR), were of analytical grade.

Cells

Three different strains of the EAC were used. (1) An original, hyperdiploid, EAC strain, kindly supplied by H. Lettré to A. Graffi in 1948. This strain has since been transplanted weekly i.p. to female AB, later to ICR mice (random). (2) Another original EAC strain changed to tetraploidy by permanent *in vitro* cultivation. In this state these tumour cells were unable to proliferate sufficiently in our test system so that we could use it only for preparation of the inhibitor. After 10 weekly passages *in vivo* we detected that the tumour changed its ploidy to hyperdiploidy within the following three passages. Afterwards it was able to proliferate *in vitro* under our conditions, and we tested its sensitivity to the purified inhibitor. (3) The hyperdiploid, colchicin resistant mutant EAC substrain HD33. This substrain was selected in Heidelberg (Deutsches Krebsforschungszentrum [19–21] from a mutant strain obtained by treatment with *N*-methylcolchicamide [22, 23]. Both, the second and third strain were kindly supplied by B. Puschendorf, Institut for Medicine, Chemistry and Biochemistry, University of Innsbruck, in 1983, and we used them after adaptation to female ICR mice.

As a marker, which was checked histologically, we used the different glycogen forming capacities of the strains: while both original strains are glycogen non-storing, the mutant strain was glycogen storing. Therefore, we refer in the following to the original strains as EAC Ø-Buch and EAC Ø-Innsbruck, to the mutant strain as EAC HD33.

Glycogen staining

The presence of glycogen was proved with the PAS reaction [24, 25]. The glycogen nature of the stained material was checked with the enzyme - Amylase [26]. Results were in agreement with the literature [27].

Immunofluorescence analysis for cytokeratins

EAC cells were washed 4 times at intervals of about 2 hr with medium 199 supplemented with 10% CS in order to remove unspecifically attached mouse antibodies. Cell smears on slides were prepared from the cell pellet, fixed in formalin (5% in PBS, 5 min at room temperature) and permeabilized with digitonin (6 μg/ml in PBS). Indirect immunofluorescence tests were performed using monoclonal anti cytokeratin antibodies A45-B/B3 [28], A51-B/H4 (not published) and PKK-1 (Labsystems Oy, Helsinki) and FITC labelled goat anti-mouse globulin (Staatliches Institut für Immunpräparate und Nährmedien, Berlin) diluted 1 : 60. The tests revealed the presence of a few cytokeratin filaments in many but not all ascites cells (not shown). There were no differences found between the three strains in this respect. The results confirm the epithelial origin of the cells, although the antigen expression seems to be somehow disturbed. EAC HD33 cells differed from the others in that they reacted with the FITC labelled second antibody alone showing distinct spots probably localized in the nuclei.

Methods of cell culture

The suspension cell culture was described in detail elsewhere [15]. Briefly, cells were harvested from the peritoneal cavitiy of mice (12–14 days after transplantation) and were cultured in medium 199 (4% CS, 2.5–3 × 10^5 cells/ml).

Each sample was performed in duplicate and differences were below 5% of the mean value. Each experiment was reproduced at least two times.

The purified inhibitor from bovine mammary gland and the inhibitory fractions from ascites fluid were added at the beginning of the experiments. To the controls the same volume of the appropriate buffer was added.

Assay for the effects of growth factors, CdR and preincubation of the cells on the inhibitory activity

Growth factors (EGF, insulin) or CdR and the inhibitor(s) were added under serum-free conditions at the beginning of cultivation. After 4 hr CS was added to a final concentration of 4% [17, 18].

The preincubation of cells was performed under normal culture conditions for 4 hr. After medium change cells were cultured in the presence or absence of the inhibitor.

Purification procedures

The purification procedure for the inhibitor from bovine mammary gland has been described elsewhere [29]. Most preparations revealed a single band of 13 KD by SDS gel electrophoresis.

The inhibitory activity of the ascites fluid was prepared by stepwise ultrafiltration as described earlier [30]. The protein content was determined by the method of Lowry *et al.* [31].

RESULTS

Specificity of the growth inhibitor from bovine mammary gland (GI)

As shown in Fig. 1, both EAC Ø responded to 1–100 ng GI/ml with a maximal inhibition of

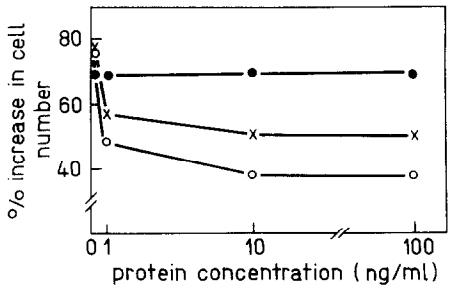

Fig. 1. Inhibitory activity of GI on growth of EAC Ø-Buch (○—○), EAC Ø-Innsbruck (x—x) and EAC HD33 (●—●).

40–50% and half maximal inhibition at about 1 ng/ml (as usually found and reported earlier [15]) whereas there is no inhibition of the EAC HD33 cells. Since cell growth was similar in both cases, this different response is not a consequence of differences in the proliferation intensity.

Specificity of EAC ascites fluid derived growth inhibitory fractions (GIF)

Figures 2A and 2B demonstrate that GIF Ø-Buch and GIF Ø-Innsbruck inhibit the growth of

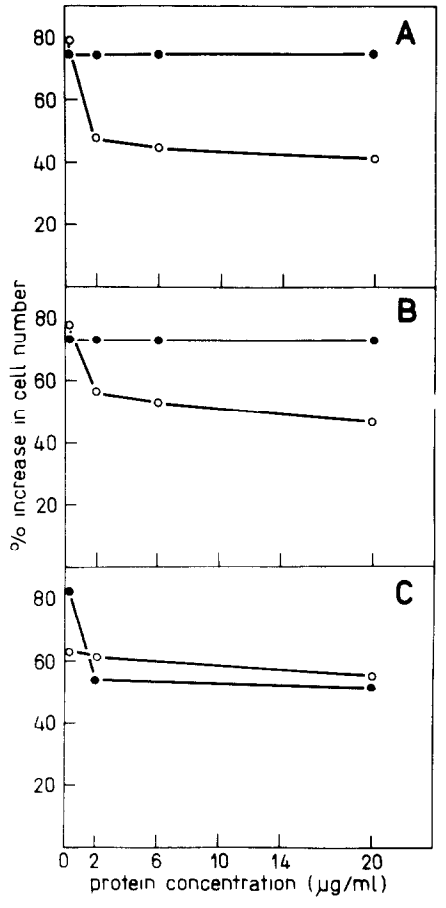

Fig. 2. Inhibitory activities of GIF's on growth of EAC Ø-Buch (○—○) and EAC HD33 (●—●). A—GIF Ø-Buch, B—GIF Ø Innsbruck and C—GIF HD33.

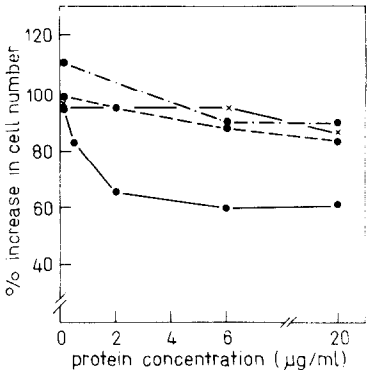

Fig. 3. Inhibitory activity of GIF HD33 on EAC HD33. ●-●, cells from stationary phase; ●--●, cells from stationary phase after preincubation; ●---●, cells from the exponential growth phase (6 days old); x-x, cells from stationary phase treated with heat inactivated GIF HD33.

EAC Ø-Buch but not that of EAC HD33 cells. Therefore, within the concentration range investigated, the two GIF from ascites fluid show the same specificity as the pure GI from bovine mammary gland. EAC HD33 cells, resistant against the inhibitors tested so far, were inhibited by GIF HD33. GIF HD33 also inhibited, though less effectively, EAC Ø cells. Cytotoxic effects of all the GIF's were not observed by Trypan blue staining.

Further characterization of GIF HD33

As described earlier, GI is characterized by the fact that its activity is dependent on the growth state of the target cells (not active on exponentially growing cells and after preincubation of stationary cells with 4% serum) and can be antagonized by EGF, insulin or 2'-deoxycytidine [15, 17, 18]. Also GIF Ø-Buch has been shown to be dependent in its activity in the same way on the growth state of the cells and to be antagonized by insulin [16]. In the following we studied GIF HD33 and, so far not already done, GIF Ø-Buch with regard to the properties typical for GI. Figure 3 shows that GIF HD33 acts less well on preincubated or exponentially growing cells, but some residual effect is found also on these cells. GIF Ø and GI are completely inactivated by a treatment of 80° C for 12 min [15, 16]. The same treatment strongly decreases GIF HD33 activity (about 10% residual activity in three experiments).

Furthermore it was found that the activity of GIF HD33 on EAC HD33 cells is fully overcome by EGF (10 ng/ml), insulin (10 ng/ml) and 2'-deoxycytidine (10^{-5}M) in the same concentration range as described earlier for GI [15, 17, 18] (Fig. 4). The same is true for the effect of GIF Ø-Buch on EAC Ø-Buch cells with regard to EGF and 2'-deoxycytidine (Fig. 4) (for insulin already reported earlier [16]).

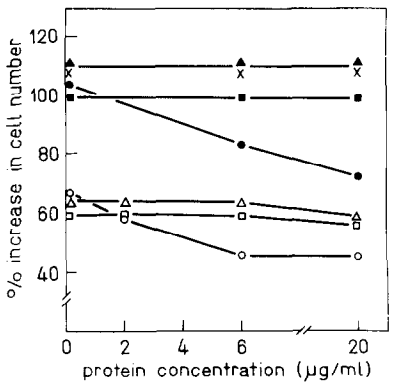

Fig. 4. Effects of 2'-deoxycytidin, insulin and EGF on the inhibitory activity of GIFs. I. *GIF HD33 on EAC HD33.* ●—●, *no other additions*; ▲—▲, 10^{-5}M *2'-deoxycytidine*; x—x, 10 ng/ml *insulin*; ■—■, 10 ng/ml *EGF.* II. *GIF Ø Buch on EAC Ø Buch.* ○—○, *no other additions*; △-△, 10^{-5}M *2'—deoxycytidine*; □—□ 10 ng/ml *EGF.*

DISCUSSION

Table 1 summarizes the activities of the different inhibitors on EAC Ø Buch and EAC HD33 (EAC Ø Innsbruck responding for GI in the same way as EAC Ø Buch). The insensitivity of the HD33 cells against the highly purified inhibitor from bovine mammary gland and against the inhibitory activities of the ascites fluid of the original strains was unexpected and shows that selectivity of such inhibitors can be artificially changed in a way which does no longer correspond to a "tissue specificity" (as e.g. called for by the chalone concept). The insensitive mutant substrain has been obtained by treatment of the original strain with *N*-methylcolchicamide [19, 20, 22]. Though initially it grew faster than the original strain (killing the animals earlier), it did no longer differ in this respect when checked again 16 years later [23]. Besides its glycogen storing properties it differs from the original strain by its colchicine resistance, and several other parameters, such as antigenicity, and membrane properties [32–35]. Thus, the treatment with the mutagen has resulted in profound perturbations in the genetic material and its expression. From our results one might suggest that also the autocrine inhibitor produced by the cells as well as the site the inhibitor acts upon have been altered in comparison to that of the original strain.

It is difficult to explain, however, that both inhibitor and "receptor" should have been altered separately (e.g. by single-step mutations) in a meaningful way. Colchicine resistance in EAC HD33 cells is due to an impaired penetration of the compound into the cell [20]. This type of resistance has been found in other cases to be caused by amplification of genes [36–39] whose products build up a membrane barrier for colchicine and other agents. Such gene amplification may also influence expression in other parts of the genome, or other genes particularly in the neighbourhood may be co-amplified. Thus, a switch in EAC HD33 to another regulatory system may have occured, e.g. in a way corresponding to an altered state of differentiation [37]. From the myelopoietic system it is well known that different states of differentiation respond to different growth inhibitors [3,5]. It is an open question whether changes in selectivity as here observed after mutagen treatment may occur spontaneously within the expected variations of cell strains over a long time (especially when transplanted or cultivated in different laboratories under different conditions such as mouse strains for the *in vivo* passage or media for the culture). The fact that the two glycogen non-storing strains, kept for 37 years in different laboratories (Buch and Innsbruck/Heidelberg) show the same specificity, even though changes of polyploidy did occur in this time (see Methods), is not in favour of a spontaneously altered selectivity. On the other hand, the results point to the possibility that treatment with mutagens *in vivo*, as e.g. cytostatic agents, often producing similar resistance phenomena, or carcinogens may alter the autocrine regulation of cell proliferation. This could even play a role for pathological processes as the malignant transformation.

Table 1. Comparison of the different growth inhibitory activities on EAC Ø Buch and EAC HD33

	GI	GIF Ø Buch	GIF Ø Innsbruck	GIF HD33
EAC Ø Buch	+	+	+	(+)
EAC HD33	−	−	−	+

All three inhibitors are antagonized by insulin, EGF and 2'-deoxycytidine. The antagonism with the latter compound has been discussed earlier for the factor from bovine mammary gland as evidence that in one way or other ribonucleotide reductase is involved in the inhibitor action [18]. The same conclusion can now also be drawn with regard to the factors from the ascites fluids. In the context of the present paper, these results indicate that the three factors differ in the way they interact with the cells but not in the reactions they induce in consequence of this interaction.

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