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Amino acid uptake in plasma membrane vesicles isolated from proliferating tumor cells and tissues

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Summary. The transport of L-alanine, a natural substrate of system A, across plasma membrane vesicle preparations has been studied in the early stages of rat DENA-PH hepato-carcinogenesis and in a very undifferentiated rat ascites hepatoma cell line (Yoshida AH-130) in the exponential and stationary phase of growth.

Kinetic analyses indicated an increase of the V_{max} value in DENA-PH-treated rats 30 h after partial hepatectomy as well as in exponential growing Yoshida ascites cells. In DENA-PH-treated rats the Km value was drastically reduced 7 and 60 days after surgery, when enzyme-altered hyperplastic and preneoplastic lesions were present in rat liver. Drastically reduced Km values were also found in Yoshida ascites cells.

The results suggest that an altered alanine transporter might take place in liver plasma membranes from carcinogen-treated rats. This appears to occur also in an established tumor cell line, grown in vivo.

Keywords: Amino acids – Alanine transport – Plasma membrane vesicles – System A-Yoshida hepatoma – Hepatocarcinogenesis

Abbreviations: AAF, 2-acetylaminofluorene; DENA, diethylnitrosamine; PH, partial hepatectomy; PMSF, phenylmethanesulfonyl fluoride.

Introduction

Nutrient transport plays an important role in the regulation of cellular metabolism and growth (Kilberg, 1986; Guidotti *et al.*, 1978). An increase in transport rates of essential nutrients (amino acid and hexose) in response to proliferation has been reported (Boerner and Saier, 1982, Borghetti et al., 1980; Foster and Pardee, 1969; Isselbacher, 1972).

Since the plasma membrane acts both as a permeability barrier and as a transducer of biological information, cellular membrane modifications of tumor

cells have been considered as one of the main aspects of neoplastic transformation (Friedman and Skehan, 1981).

Alterations in the plasma membrane may directly or indirectly involve transport function.

System A transport for neutral amino acids is regulated by a wide variety of external stimuli and its activity is well correlated with the cellular growth state (Boerner and Saier, 1982, 1985; Guidotti et al., 1978; Handlogten and Kilberg, 1988). Recently, this transport system has been indicated as a possible target of oncogene action (Dawson and Cook, 1987; Saier et al., 1988).

Most of the studies on amino acid transport in transformed or proliferating cells have been carried out in whole cells (Boerner and Saier, 1982; Borghetti et al., 1980; Foster and Pardee, 1969; Isselbacher, 1972), were the analysis of the transport mechanism is complicated by cellular metabolism.

Transport experiments performed with vesiculated plasma membrane fragments make it possible to assay the transport activity separated from the effects of intracellular metabolism and compartmentalization, and permits the use of physiological substrates instead of non-metabolizable analogs.

In this work alanine transport, a natural substrate for system A, has been studied in the early stages of rat DENA-PH hepatocarcinogenesis (Solt et al., 1977) and in a very undifferentiated rat ascites hepatoma cell line grown in vivo (Yoshida AH-130) using purified preparations of plasma membrane vesicles.

Materials and methods

Chemicals

L-(U-^{14}C)-Alanine 150 mCi/mmol was purchased from Amersham International (Amersham, UK). Percoll was purchased from Pharmacia (Uppsala, Sweden). All other reagent were analytical grade products from BDH (Italy).

DENA-PH hepatocarcinogenesis: animals and treatments

Fischer F344 male rats weighing 125–150 g, housed in a light controlled room (lights on from 7.00 to 19.00) at a temperature of 23 ± 1°C, were maintained on a high (24%) protein semi-synthetic diet (Piccioni, Brescia, Italy) and water ad libitum. Animals were acclimatized for 1 week before beginning the experiment and then injected intraperitoneally with 200 mg/kg DENA dissolved in saline (100 mg/ml). A non-treated control group (NTC), maintained on the high protein diet, was also prepared.

Two weeks later the injected rats were placed on a diet containing 0.02% AAF (Piccioni, Brescia, Italy) to inhibit growth of normal hepatocytes, following partial hepatectomy.

At the 7th day of AAF treatment animals were given light ether anesthesia and partial hepatectomy (67%) was performed between 9 and 11 a.m. The animals were maintained on the AAF diet until sacrifice. One week after operation the remaining animals were returned to the AAF-free basal high protein diet.

Animals were killed 30 h, 7 and 60 days after operation, and the livers were used immediately.

Yoshida ascites hepatoma: cell growth and harvesting

Yoshida ascites hepatoma cells (AH 130) were propagated by injection (50–60 × 10^6 cells) into the peritoneal cavity of male Wistar rats, maintained in a light controlled room (lights on from 7.00 to 19.00) at a temperature of 23 ± 1°C.

Exponential growing and stationary phase ascites cells were harvested on day 4 and 12 after inoculation (Comolli et al., 1984, 1986). Cells were washed to remove the ascitic fluid and contaminating erythrocytes with a washing medium of the following composition: 35 mM Tris-HCl buffer, pH 7.6, 146 mM NaCl (medium A). Cells were counted and stored in medium A with 10% glycerol at −80°C in 2.5 ml aliquots containing 100×10^6 cells/ml. Before use, cells were thawed rapidly at 37°C , centrifuged, and resuspended in 2 mM HEPES-Tris buffer, pH 7.5, 250 mM sucrose, containing 0.1 mM PMSF (medium B).

Preparation of plasma membrane vesicles

The plasma membrane vesicles from rat liver were prepared according to the Epping and Bygrave (1984) method with minor modifications (Leonardi et al. 1988), those from the Yoshida ascites tumor with a procedure based on a modification (Leonardi et al., 1990) of the Boumendil-Podevin and Podevin (1983) method.

Protein determination

Protein determinations were carried out according to Bradford (1976), with a kit from Bio-Rad (Richmond, CA), using bovine serum albumin as standard.

Transport assay

The transport experiments were performed at 25°C by rapid filtration technique in quadruplicate with an automated device. 10 μl of the vesicle suspension (at a protein concentration of 3–5 mg/ml) was added to 10 μl of an incubation mixture containing 250 mM sucrose, 5 mM HEPES-KOH or 2 mM HEPES-Tris pH 7.5, L-[U-^{14}C]alanine and salt gradients as necessary to obtain the final concentrations indicated in the legends of the figures. At selected times, samples were diluted with 2 ml of ice-cold stop solution (250 mM sucrose, 5 mM HEPES-KOH or 2 mM HEPES-Tris pH 7.5, 100 mM NaCl), filtered through a prewetted 0.45 μm pore size cellulose-nitrate filter (Micro Filtration Systems, Dublin, CA) and rapidly washed with 3 ml of ice-cold stop solution. The filters were then dissolved in a scintillation mixture and the radioactivity counted in a liquid scintillation spectrometer (Packard, Model 300C).

Typical experiments are reported in the figures (obtained from one membrane preparation). The results are consistent with those observed from at least one other preparation.

Results and discussion

We have examined and compared the kinetic of Na^+-dependent alanine uptake, a natural substrate of system A, across plasma membrane vesicle preparations isolated from intact rat liver or during the early stages of rat DENA-PH-hepatocarcinogenesis (Solt et al., 1977) and from a very undifferentiated rat ascites hepatoma cell line grown in vivo (Yoshida AH 130).

In DENA-treated rats hepatocytes were initiated with a relatively large dose of the carcinogen (200 mg/kg body weight). AAF, a "selection" agent capable of suppressing the growth of normal hepatocytes, was then administered for two weeks and in the middle of this treatment the animals were subjected to partial hepatectomy to actuate rapid growth of DENA altered hepatocytes not suppressed by AAF. Rats were sacrificed at different times after surgery (30 h, 7 and 60 days) and plasma membrane vesicles were prepared from rat liver.

The initial alanine uptake as a function of external alanine concentration between 30 μM and 5000 μM, measured in the presence of a NaSCN gradient at 6 s of incubation showed a relevant linear component added to a saturable

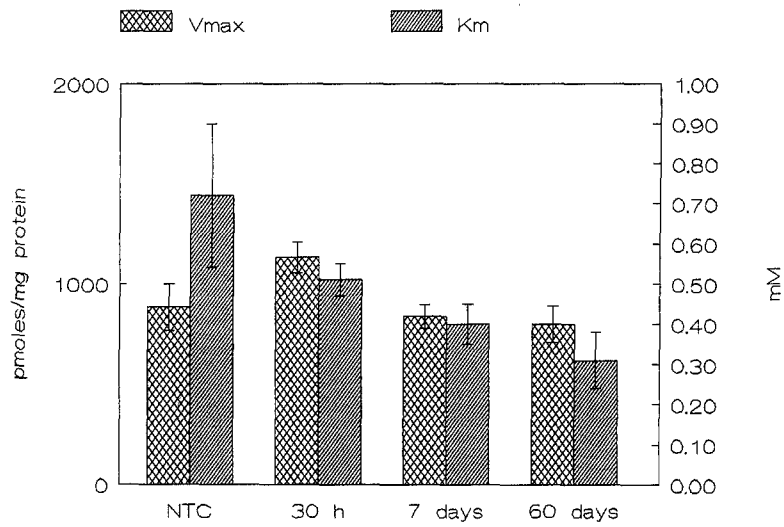

Fig. 1. Kinetic parameters of alanine transport in Fischer 344 rat liver plasma membrane vesicles isolated from non-treated controls (NTC) and from DENA-injected animals at different times after partial hepatectomy. Experimental conditions: liver plasma membrane vesicles resuspended in 250 mM sucrose, 5 mM HEPES-KOH pH 7.5 and incubated in 250 mM sucrose, 5 mM HEPES-KOH pH 7.5, 100 mM NaSCN and 0.03–5 mM L-[^{14}C]-alanine (final composition). The uptakes were terminated after 6 s of incubation. The kinetic parameters were calculated by non-linear regression analysis according to Bianchi et al. (1988). Kinetic parameters are expressed ± S.D.

one. The mean values of the kinetic parameters of the transport are reported in Fig. 1; they were calculated by non-linear regression analysis of the data as described by Bianchi et al. (1988).

The kinetic parameters of DENA-injected rats differed from those found in liver plasma membrane vesicles obtained from non-treated control animals. Particularly, 30 h after partial hepatectomy the maximum velocity (V_{max}) was markedly increased as compared with non-treated controls, with no changes in Km values. This could be related to the stimulation of cellular division after partial hepatectomy. In fact, an increase of the V_{max} value in response to mitogens has been described (Borghetti et al., 1980; Foster and Pardee, 1969; Oxender and Cecchini, 1977). At 7 and 60 days after surgery, when the stimulatory effect of partial hepatectomy almost or totally disappeared and enzyme-altered liver foci and nodules were present in rat liver (Comolli et al., 1989) the V_{max} values decreased and correlated well with those of non-treated controls. In contrast, the affinity of the transporter for the amino acid was found to increase progressively.

The gradual decrease of the Km value observed in DENA-injected rats at different times after partial hepatectomy could be related to the appearance of a high-affinity transporter in concomitance with the appearance of hyperplastic and preneoplastic nodules in rat liver under these conditions.

The existence of a system A with high-affinity and inherent differences has been observed in various transformed cells (Boerner and Saier, 1982; Dudek et al., 1987; Handlogten et al., 1981; Handlogten and Kilberg, 1988).

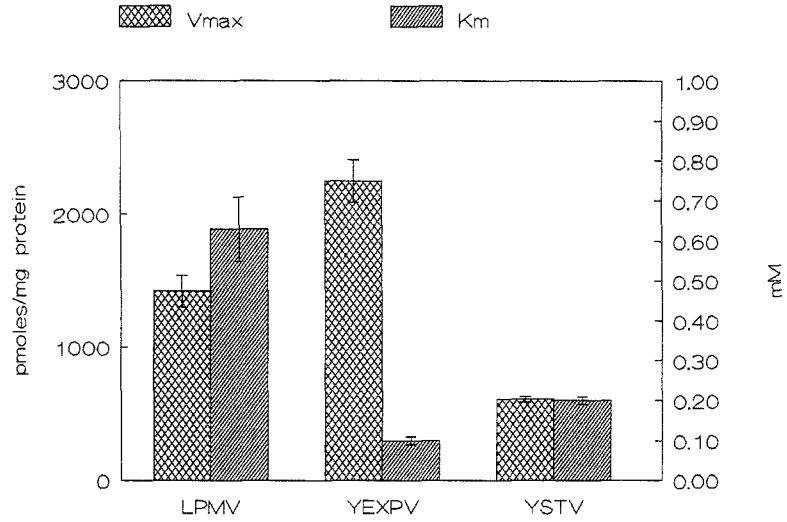

Fig. 2. Kinetic parameters of alanine transport in plasma membrane vesicles isolated from the liver of Wistar rats (LPMV) and from exponential (YEXPV) and stationary (YSTV) Yoshida ascites hepatoma cells. Experimental conditions: plasma membrane vesicles resuspended in 250 mM sucrose, 2 mM HEPES-Tris pH 7.5 and incubated in 250 mM sucrose, 2 mM HEPES-Tris pH 7.5, 100 mM NaSCN and 0.01–1 mM L-[^{14}C]-alanine (final composition). The uptakes were terminated after 6 s of incubation. The kinetic parameters were calculated by non-linear regression analysis according to Bianchi et al. (1988). Kinetic parameters are expressed ± S.D.

Fig. 2 reports the mean values of the kinetic parameters of alanine transport in the two growth phases, exponential and stationary, characterizing the in vivo growth of Yoshida rat ascites hepatoma cells. The kinetic parameters were calculated by non-linear regression analysis (Bianchi et al., 1988) from the kinetics of alanine uptake as a function of external amino acid concentration between 10 μM and 5000 μM, measured in the presence of a NaSCN gradient at 6 s of incubation.

The kinetics of alanine transport across the plasma membrane vesicles isolated from these tumor cells was characterized by a reduced Km and, only in the exponential phase of growth, by an elevated V_{max} (Leonardi et al., 1990).

The results here reported suggest changes of alanine transport (e.g. system A) in the conditions studied related both to mitogenic and transforming stimuli. The enhanced maximum velocity in proliferating cells could indicate an increase in the number of transporters for this amino acid. In contrast the changes in the affinity constant of the transporter during the early stages of DENA-induced hepatocarcinogenesis and in the ascites hepatoma cells point to the appearance of a high-affinity transporter in response to carcinogenic stimuli.

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