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ENKEPHALINASE ACTIVITY IN THE INTESTINAL EPITHELIAL CELLS OF THE FETUS OF 19 DAYS AND THEIR IMMORTALIZED AND TRANSFORMED COUNTERPARTS THE SLC-CELL LINES

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Enkephalinase (EC 3.4.24.11), an enzyme widely distributed in brain and peripheral tissues of human and various animal species, was measured in the intestinal fetal cells and in the intestinal epithelial cells of adult rat, where its activity was respectively $96,1 \pm 10,18$ fmol/mg protein and $52,27 \pm 8,43$ fmol/mg protein. The immortalized cell lines: SLC-11 (after transfection with the plasmid containing oncogene from the human adenovirus type 2-E1A), SLC-21 (plasmid containing oncogene from polyoma virus) and SLC-41 (plasmid containing oncogene from simian virus 40 large tumor antigen) presented relatively strong enkephalinase activity; it was respectively $28,3 \pm 1,7$, $37,9 \pm 3,6$ and $49,3 \pm 3,1$ fmol/mg protein. The cells of SLC-12T and SLC-44T lines, obtained after transfection with the mutant Ha-ras-1-gene and possesing tumorigene potency have the enkephalinase activity very decreased: $1,6 \pm 0,9$ and $8,7 \pm 3,2$ fmol/mg protein ($p < 0,001$). This interesting properties of the tumorigene cells may constitute a new subject of investigations in the carcinomas therapy.

K e y w o r d s : *enkephalinase, neutral endopeptidase, membrane-bound endopeptidase, immortalized cells.*

INTRODUCTION

Enkephalinase (EC 3.4.24.11), a membrane-bound metalloendopeptidase also designated as a neutral endopeptidase (NEP) is an enzyme widely distributed in brain and peripheral tissues of human and various animal species (1—4). Orginally purified from rabbit kidney, where it is the most abundant (5), NEP is also largely present in the epithelial cells of the gastrointestinal tract, namely in the intestinal cells (1,2,6—13). However, the physiological role of enkephalinase hasn't been definitively clear, this membranebound metalloendopeptidase is largely responsible for the inactivation not only of en-

dogenous enkephalins, but also of other biologically active peptides: atrial naturiuretic factor (ANF) (14,15), gastrin (16,17), vasoactive intestinal polypeptide (VIP) (18), cholecystokinin (12,16,19), substance P (8,11, 20—23), gastrin releasing peptide-10 (7,8), neurotensin (9,21,24,25) and tachykinins (22,26). In the human gastrointestinal tract, the highest enkephalinase activity was observed in doudenal and jejunal epithelial cells (27). The intestinal mucosa is characterised by an extremely fast reneval of the normal epithelial cells. Their mitogenic activity, morphological and enzymatic properties, mechanisms of proliferation and differentiation are the very interesting subject of investigations in primary culture. The models of permanent intestinal epithelial cells in culture that would possess a high proliferative potential and would be exempt from the neoplastic transformation after transfection with a known oncogen were proposed by Emami et al. (28).

In the present study we would like to present enkephalinase activity in normal intestinal cells isolated from rat fetuses at 19 days of gestation and in their counterparts transfected using three different recombinat plasmids containing the viral oncogene: E1A of adenovirus type 2 (Ad2) and large tumor (T) antigens of polyoma virus (py) and simian virus 40 (SV40). The resulting transfected cells were designated SLC-11, SLC-21 and SLC-41, respectively (28). The SLC-12 cells line was derived from SLC-11 cells after the cloning and transfection with the mutant Ha-ras-1-gene (SLC-127) (28). Respectively, SLC-44 cells were cloned from SLC-41 cells and transfected with the mutant Ha-ras-1-gene (SLC-44T) (28). The obtained cellular lines differ in their tumorigenicity, mutant Ha-ras-1-gene transfected cells being more tumorigene in athymic nude mice (28,29).

MATERIALS AND METHODS

Intestinal cells preparation

Small intestinal epithelial cells were obtained from adult Wistar rat and Wistar rat fetuses at 19 days of gestation by previously described procedures (30). The small intestines obtained from adult rats after decapitation were opened along the lumen, a single intestine was excised at the adult stage. Rat fetuses were removed by hysterectomy while the mother was maintained under anaesthesia with sodium pentobarbital i.p. (4 mg/100 g body mass). Fetuses were bled by decapitation and section of the umbilical vein. The small intestines immersed in chilled Krebs-Ringer Phosphate (KRP) buffer during collection were excised under a dissecting microscope and mechanically cut into square fragments 1 mm long by a tissue chopper (Mc Illwain, England). The material was washed twice in ice-cold KRP buffer (pH = 7,5) containing 118 mM NaCl, 5 mM KCl, 1,2 mM $MgSO_4$, 1,2 mM KH_2PO_4 and 10 mM Na_2HPO_4. Pools of whole small intestines from 8—30 fetuses at 19 days of gestation were used for each assay.

Intestinal epithelial cells were isolated according to the modified version of the method that uses EDTA to dissociate the mucosa. Intestinal fragments were incubated for 15 minutes at 4°C in a solution containing 4,5 mM EDTA and 0,25 M NaCl (pH = 7,5). Intenstinal cells were obtained

by gentle shaking for 10 sec 20 times by hand. After each period of shaking, intestinal sections were placed in 10—40 ml fresh medium. Cells were pelleted by 2-min centrifugation (200 × g, 4°C) and washed three times with KRP buffer.

Intestinal cells transfection

The isolated cells were then washed and resuspended in Ca^{2+} — free minimal Eagle medium (S-MEM, GIBCO) containing 0,5% heat-inactivated fetal bovine serum (FBS, Biological Industries, Beth Haemek, Israel), 100 units of penicillin per ml and 100 μg of streptomycin per ml. Then the cells underwent electropermeabilization and were successfully transfected by three recombinant plasmids containing the cloned oncogenes from the human adenovirus type 2 early region E1A (SLC-11 cells) and polyoma virus and simian virus 40 large T tumor antigenes (SLC-21 and SLC-41 cells) as described previously (28). SLC-12 cells line derived after the cloning from SLC-11 cells whereas the SLC-12T cells were obtained after transfection with the mutant Ha-ras-1-gene. Respectively, SLC-44 cells were cloned from SLC-41 cells and transfected with the mutant Ha-ras-1-gene (SLC-44T) (28, 29).

Measurement of enkephalinase activity

Samples (50 μl) of the pellet suspension in the TRIS buffer (pH = 7,4) were incubated with the labelled radioactive enkephalinase substrate for 60 minutes at 25°C. Incubation was started by addition of 50 μl of the pellet suspension to the labelled substrate: [³H-D-Ala-2,Leu-5-]-enkephalin (Commissariat à Enérgie Atomique, Saday, France) (40mM final concentration) and 50 μl of TRIS-HCl buffer (pH = 7,4) containing bestatin 10 μM final concentration (Sigma, St. Louis, MO, USA) being an aminopeptidase inhibitor, and captopril (Laboratories Squibb) — an angiotensin converting enzyme inhibitor (1 μM final concentration).

Thiorphan, a selective inhibitor of the enkephalinase activity, was added to paralled incubation instead of TRIS-HCl buffer, in the final concentration of 1 μM (Sigma, St. Louis, MO, USA).

The metabolites of labelled substrate [³H-D-Ala-2,Leu-5]-enkephalin were isolated from the intact enkephalin by chromatography on a polystyrene bead column according to the method of Llorens et al. (3).

Enkephalinase activity was calculated as difference between the radioactivity of the mixture without thiorphan and the mixture with this inhibitor (3).

Measurement of protein was made by the method of Bradford.

The statistical analyse was performed using t-Student test.

RESULTS

The EC 3.4.24.11 activity was the highest in the intestinal fetal cells and it was 96,1 ± 10,18 fmol/mg protein (n = 9).

In the intestinal epithelial cells of adult rat this activity was 52,27 ± 8,43 fmol/mg protein (n = 6).

The immortalized intestinal cells lines SLC-11, SLC-21, SLC-41 and SLC-44 presented relatively strong enkephalinase activity which significantly decreased when the degree of tumorigenicity was stronger after transfection with the mutant Ha-ras-1-gene in the lines SLC-12T (p < 0,001) and SLC-44T (p < 0,01). (Tab. 1).

68

Table 1. Enkephalinase activity in the immortalized cells of different lines

Line of immortalized cells	Number of determinations	E.C.24.11 activity fmol substrate hydrolyzed min/mg (mean ± SD)
SLC-11	19	28.3 ± 1.7
SLC-12	8	4.7 ± 1.5
SLC-12 T	6	1.6 ± 0.9
SLC-21	4	37.9 ± 3.6
SLC-41	4	49.3 ± 3.1
SLC-44	8	41.6 ± 3.8
SLC-44 T	6	8.7 ± 3.2

DISCUSSION

The exploration of the expression of EC 3.4.24.11 during the ontogeny of the small intestine showed its presence during the early gestation period in human and at different stage of the intestinal development in rat and rabbit. As demonstrated by Lecavalier et al. (31), the EC 3.4.24.11 activity measured in the suckling animal is almost twice the measured in the fetal tissue. In our study, we showed the stronger activity of enkephalinase in the rat fetal small intestine at 19 days of gestation, although it is also very high in the adult rat intestine. Probably, EC 24.11 can be involved in terminal hydrolysis of smaller peptides, besides other intestinal neutral endopeptidases, according to the model proposed by Guan et al. (10) which could be a significant alternative pathway for the protein hydrolysis instead of pancreatic proteases.

The immortalized cell lines retained several of the phenotypes observed in their parent cells of the intestinal mucosa: cytoplasmic villin, cytokeratins, cells surface receptors, including also enkephalinase activity (28). This activity in the immortalized cell lines seems to be a very interesting subject for investigations. The SLC-11 cell line, similarly to SLC-21 and SLC-41 possessing relatively strong enkephalinase activity, do not have capacity of the tumor formation in athymic nude mice (28). However, the SLC-12 cell line derived from SLC-11, and SLC-44 cloned from SLC-41, after transfection by the mutant Ha-ras-1-gene transformed in SLC-12T and SLC-44T, showed much lower EC 24.11 activity than their pattern cells. Those cell lines are able to tumorigenesis in athymic nude mice (data not shown). Mutated Ha-ras-1-gene does not require a cooperating gene to trigger maligant conversion and ras genes may be involved in the process of tumorigenesis at an earlier stage than previously suspected (29).

It is known, that utilisation of specific sonds shows a great frequency of the mutation of proto-oncogenes of ras-family in human colorectal cancer. The expression of the oncogenes of ras-family are often emphasized in case of colorectal cancers and adenomatic polypes (32).

We observed an important difference of enkephalinase activity depending of oncogene used for cells transfection and in result, capacity of cell line to the tumorigenesis after the malignant conversion. The activity of EC 24.11 decreased when the tumorigene potency of cellular line increased. In our study on human colorectal cancer cell line HT-29 this activity was even undetectable at all (data not shown).

This various comportement of enzymatic activity of EC 24.11 in transformed and malignant cell lines is worth to be assayed. Some cancer tumors with small carcinoma cells (lung carcinoma, or colorectal carcinomas) or the endocrine tumors of the rectum have been shown to contain the opioid peptides and their receptors (33, 34). Enkephalins, among other biologically active peptides, have been shown to be internalized into their target cells through interaction with specific receptors, also when conjugated with the antitumor drugs, so it may constitute a new class of specific carriers against tumor cells (33). In this case, the role of enkephalinase may be very important but it requires further exploration.

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