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Characterisation of enkephalinase (EC 3.4.24.11) activity on various leukemic cells expressing the common acute lymphocytic leukemia antigen (CALLA)

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The deduced amino acid sequences of CALLA, a cell surface marker of human acute lymphocytic leukemia, and human enkephalinase (neutral endopeptidase, EC 3.4.24.11) were recently reported to be almost identical. We show that membranes of CALLA$^+$ cells of the REH lymphoblastic cell line as well as blast cells derived from the blood or bone marrow of patients with acute lymphocytic leukemia display high enkephalinase activity. This activity was abrogated by several enkephalinase inhibitors at concentrations closely similar to those required to inhibit pure human enkephalinase. However, these compounds did not significantly modify the rate of REH cell proliferation in vitro. Hence, the functional role, if any, of the high peptidase activity in lymphoblastic cells remains to be established.

Common acute lymphocytic leukemia antigen; Enkephalinase; Neutral endopeptidase; (REH cell line, Pre-B leukemic cell)

1. INTRODUCTION

Enkephalinase (EC 3.4.24.11, neutral endopeptidase) is a 90 kDa membrane metallopeptidase initially characterized in brain [1] where its role in the inactivation of endogenous enkephalins has been established [2,3]. However, the enzyme has subsequently been found in many other tissues, particularly in kidney where a high activity is present [4], and has been shown [5–7] to be identical with a peptidase identified earlier by using the B-chain of insulin as substrate and designated 'neutral proteinase of kidney brush borders' [8]. Following its purification, enkephalinase was shown to hydrolyse a variety of exogenous neuronal or hormonal peptides besides enkephalins, although its participation in the inactivation of these other peptides in vivo largely remains to be established [9]. Recently, the cDNA sequences corresponding to the rat [10], rabbit [11] and human enzymes [12] were reported.

The common acute lymphoblastic leukemia antigen (CALLA/CD 10) is a cell surface marker in human acute lymphocytic leukemia, being expressed by pre-B-phenotype leukemic cells but not normal peripheral blood mononuclear cells. However, CALLA was also identified in a variety of nonlymphoid cells including in the kidney [13,14]. It was characterized as an approx. 100 kDa glycoprotein for which no biological activity was shown. However, the recent cloning and sequencing of a cDNA encoding for CALLA showed the deduced amino acid sequence to be strongly homologous to those of rat and rabbit enkephalinase and almost identical to that of the human enzyme [15,16].

We show here that a CALLA$^+$ cell line and lymphoblastic cells from leukemic patients express high enkephalinase activity. However, inhibition

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of the peptidase by various compounds does not apparently modify the rate of in vitro proliferation of cells from the CALLA$^+$ REH line.

2. MATERIALS AND METHODS

2.1. *Cells*

The cell line REH, originally derived from an acute lymphoblastic leukemia [17], was purchased from the American Tissue Culture Collection (ATCC, CRL 8 86 REH). Cells were grown as suspension culture in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (Seromed. Biochrom., Berlin), penicillin, and streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

Mononuclear cells from heparinized peripheral blood or the bone marrow of two patients with acute lymphoblastic leukemia were obtained by centrifugation through a Ficoll-Hypaque (Sigma, St. Louis, MO) density gradient (d = 1.077, 400 × g, 20 min). Cells (CALLA$^+$) were then washed twice with 10% (v/v) fetal calf serum. A cell suspension was prepared in 0.9% NaCl, adjusted to 5 × 10^6/ml and stored at −20°C until enzyme assays. Control mononuclear cells from healthy donors were prepared in the same way. In order to obtain non-adherent cells, i.e. lymphocytes, cells were seeded for 2 h at 37°C in tissue culture flasks (75 ml, Corning, USA) containing a humidified atmosphere (5% CO_2). Viable non-adherent cells were then collected, washed in 0.9% NaCl, resuspended in the same medium (5 × 10^6 cells/ml) and stored at −30°C until assays.

Table 1

Enkephalinase activity of various cells

Cells	Enkephalinase activity (pmol/min per 10^6 cells)
REH line	1986 ± 95
Blood mononuclear	1362 ± 39
Bone marrow	929 ± 20
Blood mononuclear cells of healthy donors	< 20

The enzyme activity was evaluated by the fluorometric assay using 25 μM Suc-Ala-Ala-Phe-AMC as substrate and defined as that inhibited by 10 μM thiorphan. Means ± SE of values from 3 experiments

2.2. *Determination of enkephalinase activity*

After thawing at room temperature, cells were homogenised in their suspension medium by sonication for 10 s. Homogenates were centrifuged (30000 × g, 20 min, 4°C) and both supernatant and pellet (resuspended in 50 mM Tris-HCl buffer, pH 7.5) used for enzyme assays.

Enkephalinase activity was evaluated on 50 μl of membrane suspensions or supernatants, using either a fluorometric [18] or an isotopic assay [19].

Briefly, for the fluorometric assay, the fluorogenic substrate was succinyl-Ala-Ala-Phe-amidomethylcoumarin (Suc-Ala-Ala-Phe-AMC, 25 μM final concentration). After 10 min incubation at 37°C, released Phe-AMC was quantitatively hydrolysed in the presence of excess aminopeptidase M and the fluorescence quantified (excitation 367 nm; emission, 440 nm).

For the isotopic assay, 40 nM [^{3}H](D-Ala^2,Leu^5)enkephalin (CEA, Saclay) was the substrate. After incubation for 30 min in the presence of 1 μM captopril, an angiotensin-converting enzyme inhibitor, and 10 μM bestatin, an aminopeptidase inhibitor, the hydrolysis product was isolated by polystyrene bead column chromatography and the radioactivity quantified by liquid scintillation spectrometry.

In both assays, blanks were obtained by parallel incubations in the presence of 0.1 μM thiorphan.

Recombinant human enkephalinase [12] was a kind gift from Bernard Malfroy (Genentech, San Francisco, USA).

Table 2

Inhibition of enkephalinase activity of human recombinant enkephalinase and CALLA$^+$ cells by various compounds

Enzyme source	IC_{50} values (nM)			
	Fluorometric assay		Isotopic assay	
	Thiorphan	Phosphoramidon	Thiorphan	Kelatorphan
Recombinant h-enkephalinase	1.5 ± 0.1	2.0 ± 0.1	1.4 ± 0.2	2.2 ± 0.1
REH cell line	1.7 ± 0.2	2.3 ± 0.4	0.7 ± 0.1	1.8 ± 0.2
Mononuclear blood cells from a leukemic patient	2.2 ± 0.3	2.5 ± 0.3	ND	ND

Enzyme activities were evaluated either on 1 ng recombinant enzyme and 10^5 cells using 25 μM Suc-Ala-Ala-Phe-AMC (fluorometric assay) or on 10 ng recombinant enzyme and 10^7 cells using 40 nM [^{3}H](D-Ala^2,Leu^5)enkephalin (isotopic assay) as substrate. Maximal inhibition was 98 ± 2% with the various enzyme sources and inhibitors. Values are means ± SE calculated by least-squares analysis of concentration-inhibition plots established using at least five concentrations of the inhibitors and triplicate assays

Table 3

Daily cell counts of REH cells cultured in the presence or absence of enkephalinase inhibitors (number of cells: $\times 10^{-5}$)

	Controls	Phosphoramidon (10 μM)	Kelatorphan (10 μM)
Day 1	1.99 ± 0.14	1.90 ± 0.21	1.80 ± 0.23
Day 2	2.83 ± 0.22	2.95 ± 0.16	2.99 ± 0.49
Day 3	4.65 ± 0.50	4.57 ± 0.37	4.54 ± 0.56

Cells (10^5 per ml at day 0) were suspended either in RPMI-1640 medium supplemented with 10% fetal calf serum or in the same medium containing 10 μM phosphoramidon or kelatorphan and were counted every day for 3 days. Means ± SE of data from 3 experiments

3. RESULTS

3.1. *Characterization of enkephalinase activity of CALLA$^+$ cells*

Membranes of all CALLA$^+$ cells tested, i.e. the REH cell line, the blood and bone marrow blast cells from two patients with established diagnosis of acute lymphoblastic leukemia hydrolysed efficiently Suc-Ala-Ala-Phe-AMC (table 1) and [^{3}H](D-Ala^2,Leu^5)enkephalin. In contrast, the hydrolysing activity was undetectable in the soluble fraction of the same cells and very weak (<20 pmol/min per 10^6 cells) in corresponding blood cells from five normal donors. The peptidase activity responsible for hydrolysis of either substrate by CALLA$^+$ cells was almost completely (~98%) inhibited by thiorphan, phosphoramidon or kelatorphan with IC_{50} values closely similar to those found with pure (recombinant) human enkephalinase (table 2). The enkephalinase activity of cultured REH cells (expressed per 10^6 cells) did not significantly differ between days 0 and 3 of culture and corresponded approximately to that of 50 ng pure enkephalinase.

3.2. *Effects of enkephalinase inhibitors on REH cell proliferation in vitro*

When REH cells were cultured in the presence of 10 μM phosphoramidon, kelatorphan (table 3) or thiorphan (not shown), their proliferation, assessed by daily cell counts, did not significantly differ from that of cells cultured in their absence.

4. DISCUSSION

The present study indicates that CALLA$^+$ lymphoblastic cells derived from either the cell line REH or the circulant blood and bone marrow of leukemic patients express strong enkephalinase activity. The latter was characterized not only by the hydrolysis of typical substrates of the peptidase but also by the inhibitory potency of various compounds, i.e. thiorphan [20], phosphoramidon [8] and kelatorphan [21,22], which was closely similar to that found on recombinant human enkephalinase. These results could be expected from the virtual identity of the sequences of the human enzyme [12] and the CALLA antigen [15,16] but indicate, in addition, that the post-translational modifications of the protein in lymphoblastic cells are adequate to provide it with potent peptidase activity. In particular, since the peptidase requires a tightly bound zinc atom to express enzyme activity [23,24] this suggests that the level of Zn^{2+} in the cells (or culture media) was sufficient for the biosynthesis of a fully active enzyme.

While this work was in progress, Shipp et al. [25] recently reported that a CALLA$^-$ murine myeloma cell line when transfected with CALLA cDNA exhibited potent enkephalinase activity, also indicating that expression of the antigen was associated with the typical peptidase activity.

The role, if any, of the peptidase in proliferation, differentiation or functions of lymphocytes is still a matter of conjecture. The present study indicates that its inhibition did not result in any significant change in the rate of in vitro proliferation of a lymphoblastic cell line but does not exclude the possibility that the enzyme plays a critical role during lymphocyte differentiation. Our observation that normal mature lymphocytes do not display measurable enkephalinase activity is consistent with previous observations showing that CALLA is only expressed by early normal lymphoid progenitors [26–28]. This may suggest that the membrane-bound peptidase may act at a transient stage of lymphoid cell differentiation by activating or inactivating a still unidentified extracellular peptide messenger. However, it is noteworthy that long-term treatments with enkephalinase inhibitors have thus far not disclosed any significant change in lymphoid tissues or blood lymphocyte counts in several animal species (Coquet, B. and Chipkin, R., personal communications).

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