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Elevated phenylalanine levels interfere with neurite outgrowth stimulated by the neuronal cell adhesion molecule L1 in vitro

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Abstract **Elevated levels of phenylalanine (Phe) as observed in patients with phenylketonuria interfere with proper neuronal development, leading to severe psychomotor deficits and mental retardation. We have analyzed the effects of Phe on neurite outgrowth in vitro. When expressed in fibroblasts, the neuronal cell adhesion molecules L1 and plexin B3 strongly increase the length of neurites emanating from cerebellar neurons in co-culture experiments. Elevated Phe blocks L1-mediated, but not plexin B3-mediated outgrowth, whereas tyrosine is ineffective. Elevated Phe also interferes with aggregation of fibroblasts overexpressing L1, suggesting that the pathological effect of elevated Phe occurs by interfering with L1-mediated cell adhesion.**

Keywords: Plexin B3; Cell adhesion; Neurite outgrowth

1. Introduction

Phenylketonuria (PKU) is the most common monogenic disorder of amino acid metabolism, with an incidence of about 1 in 10 000 newborn children. It is caused by deficiency of the enzyme phenylalanine hydroxylase which is responsible for conversion of the essential amino acid phenylalanine (Phe) to tyrosine. Accumulation of Phe or its oxidized metabolites during postnatal development leads to severe mental retardation. Successful treatment can be achieved by strict control of dietary phenylalanine intake. Maternal PKU represents a complication of the condition in adult patients as elevated maternal Phe during early phases of pregnancy interferes with fetal development. High maternal Phe levels during pregnancy are associated with various signs and symptoms including microcephaly and mental retardation. Hypoplasia of the corpus callosum was observed in some but not all cases [1,2]. On a microscopic level, neurons from PKU patients exhibit shorter dendrites with decreased ramifications. In addition, abnormal dendritic spine structures were observed in adult as well as maternal PKU cases [3,4].

So far it is unclear how elevated Phe levels affect brain development in utero or in the postnatal phase. Remarkably, some of the clinical features of maternal PKU such as mental retardation and hypoplasia of the corpus callosum overlap with those reported for patients with L1 spectrum disease caused by mutations in the human gene for the L1 cell adhesion molecule (*L1CAM*), the gene encoding the neuronal cell adhesion molecule L1 [5,6]. The extracellular portion of L1 contains Ig- and fibronectin repeats which mediate homophilic as well as heterophilic interactions with cell surface molecules expressed on their target cells [7]. Via these interactions L1 promotes cell adhesion and outgrowth of neuronal processes that seem to be required for proper development of neuronal circuitry [8,9].

Here we use an in vitro model for neurite outgrowth and show that elevated Phe levels interfere with L1-mediated cell adhesion.

2. Materials and methods

2.1. Tissue culture

Stably transfected 3T3 fibroblast cell lines expressing *L1CAM* and Plexin B3 have been described [9,10]. The presence of expressed cell adhesion molecules in 3T3 cells was verified by Western blotting using antibodies pAbex2 (L1) and pAbB3-A raised against synthetic oligopeptides as described previously [9,10]. For neurite outgrowth assays, cerebellar neurons from 6-day-old mice (C57/6J) were prepared and plated on fibroblast layers as described [9,10]. After cultivation for another 24 h, cells were fixed using 4% paraformaldehyde and methanol, and stained using an antibody against the neuronal marker GAP43/neuromodulin (Transduction Laboratories, BD Heidelberg, Germany). Randomly selected microscopic fields were digitally captured using CytoVision software, and neurite length was measured using Scion Imaging software. Two exclusion criteria were applied: only neurites extending for more than one cell diameter were considered; in addition, only the longest neurite per cell was measured. For each experimental condition, at least three individual experiments were performed. In each case three individual cover slips were analyzed, with at least 50 neurons evaluated per cover slip, leading to a total number of >450 neurons per experimental condition.

2.2. Aggregation assays

Fibroblast cells were grown to 80–90% confluence; after dispersion of cells using 2 mM EDTA in phosphate buffered saline, cells were resuspended in growth media (DMEM + 10% FBS, Gibco) at a density of 1×10^6/ml. Cells were incubated in a rotating conical tube at 37 °C. The number of particles (representing individual cells and cell aggregates; see Ref. [10] for typical microscopic images) was determined

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Abbreviations: *L1CAM*; human gene for L1 cell adhesion molecule; PKU; phenylketonuria

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at various time points by removing aliquots and counting using a cell counter. The number of particles at a given time point (N_t) was compared to the number at the start of the incubation (N_0).

2.3. Statistical analysis

Results are presented as mean values ± S.D. for indicated number of experiments. Statistical significance was evaluated using Student's *t*-test.

3. Results

Cerebellar neurons were grown on feeder layers of fibroblast cells and analyzed for the length of their longest neurite. This experimental system has been used to determine the ability of cell adhesion molecules expressed on the fibroblast surface to stimulate neurite elongation. Neurons growing on non-transfected 3T3 cells exhibited a mean neurite length of about 60 μm. When the same experiment was performed on fibroblasts expressing *L1CAM* or Plexin B3, we recorded a strong increase in neurite length to 100 μm (L1) or 120 μm (Plexin B3) (Fig. 1). These changes are consistent with previous analyses [9,10] and are believed to reflect the ability of cell adhesion molecules on the fibroblast surface to mediate homophilic or heterophilic interactions with other cell adhesion molecules on the surface of the extending neurite, thus facilitating extension of neurites along the feeder cells. In order to analyze a potential effect of phenylalanine on this process, we added increasing concentrations of Phe to the culture media. It should be noted that Phe is an essential amino acid and is therefore present in the media at a basal concentration of 100 μM. Addition of 600 μM or 3600 μM Phe abolished the effect of L1 on neurite outgrowth completely, such that mean neurite length on *L1CAM* expressing feeder cells was similar to control values. Neurite extension on control 3T3 cells or on those expressing Plexin B3 was not affected by high Phe concentrations. This clearly indicates that elevated Phe specifically interferes with L1 mediated processes, but not with neurite extension per se.

Serum phenylalanine concentrations with teratogenic potential have been estimated to be larger than 360 μM in maternal PKU [11] and larger than 1000 μM in classical PKU. It should be noted however that concentrations in brain are about 50% lower than in serum [12]. Analysis of the concentration dependence of the Phe effect on L1-mediated neurite outgrowth showed a small but significant reduction of neurite length already after addition of 200 μM Phe (i.e. at a total concentration of 300 μM). The full effect was reached at a total concentration of 500 μM (addition of 400 μM; Fig. 2), showing that suppression of neurite extension by Phe in vitro occurs exactly within the range of concentrations considered to be pathological in patients with PKU and maternal PKU.

We performed the neurite outgrowth experiment in the presence of elevated levels of tyrosine (Tyr). Tyr is structurally similar to Phe, but elevated Tyr levels as observed in genetically determined tyrosinemia are not linked to mental retardation.

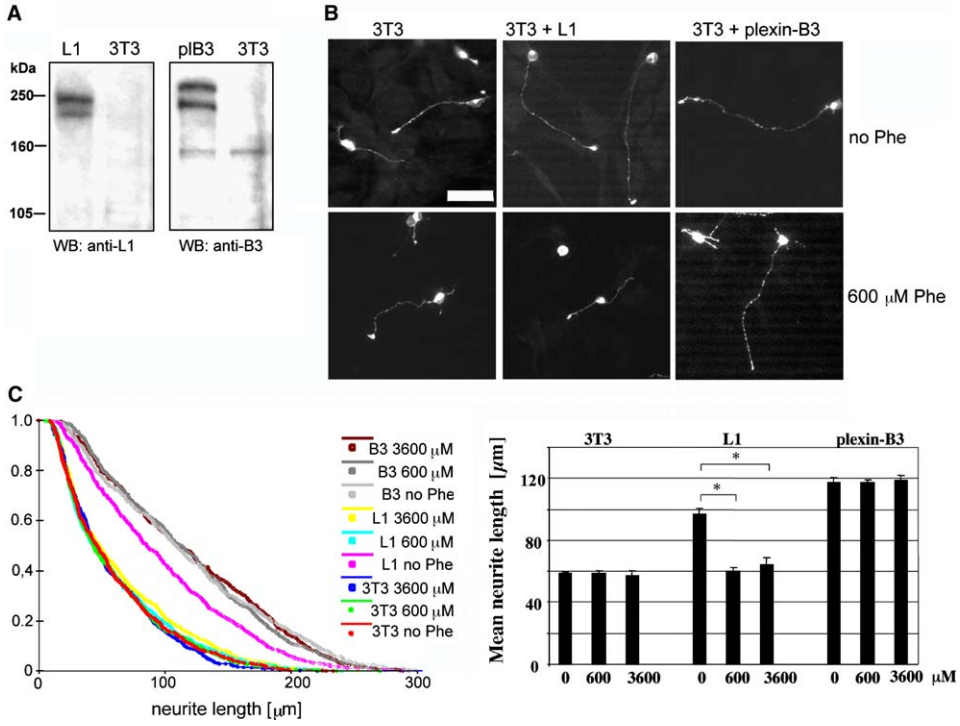

Fig. 1. (A) Expression of L1 and plexin B3 protein in NIH–3T3 cells. 3T3 cells stably expressing human L1 (L1) or human plexin-B3 (plB3), or non-transfected cells (3T3) were lysed, and identical amounts of protein were analyzed by Western blotting using anti-L1 (left) or anti-Plexin B3 (right). (B) Effect of phenylalanine on neurite outgrowth. Cerebellar neurons were plated on non-transfected (3T3) fibroblasts, or fibroblasts expressing L1 or Plexin B3, as indicated. Neurite extension was visualized after cultivation in the absence (upper panel) or presence (lower panel) of 600 μM phenylalanine. (C) Quantitative analysis of the neurite outgrowth data as shown in B. The left panel shows the distribution of neurite length obtained under different experimental conditions, as indicated. In this graph, each line contains the cumulative data of all (>450) neurons that were analyzed for the particular experimental conditions. The bar graph on the right shows the mean neurite length; each experiment was performed in triplicates of at least 50 neurons per individual experiment, and repeated three times. Bars therefore represent the mean of the mean values (±S.D.) derived from these three individual experiments, and include analysis of at least 450 neurons for each experimental condition. *, statistically significant difference, $P < 0.001$.