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Growth promotion by homocysteine but not by homocysteic acid: a role for excessive growth in homocystinuria or proliferation in hyperhomocysteinemia?

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Abstract

Excessive growth of long bones in patients with homocystinuria is still unexplained and previous work incriminating homocysteic acid could not be confirmed by others. In vitro studies from our laboratory showed that homocysteine stimulated growth in a clonogenic assay. This observation made us study plasma cyclin dependent kinase (CDK), homocyst(e)ine and homocysteic acid in 10 patients with homocystinuria and 20 controls. In addition, homocysteine and homocysteic acid were tested in a clonogenic assay to correlate the growth promoting activity with CDK. Plasma CDK (protein) correlated strongly with homocysteine ($r = 0.84$) but not with homocysteic acid. Supernatants of the clonogenic assay samples showed up to three times higher CDK levels in the presence of homocyst(e)ine but not homocysteic acid. In vitro data and the strong correlation between homocysteine and CDK suggest a role for homocysteine stimulating CDK, the starter of mitosis, with subsequent stimulation of growth. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Bone abnormalities are a cardinal symptom of homocystinurias. The skeletal disproportion is due to excessive growth of the long bones [1], although the upper/lower segment ratio of homocystinuric patients may be exaggerated by scoliosis [2]. Clopath and coworkers reported that homocysteic acid (HCA) promoted growth of hypophysectomized rats as assayed by observation of increased thickness of the epiphyseal cartilage of the tibia and by observation of tail growth. They related their findings to accelerated growth in homocystinuria, initiation of arteriosclerosis and control of cellular growth [3]. Chrzanowska and coworkers attempted to repeat these experiments using a larger number of hypophysectomized animals studying the effects of homocysteic acid on tibial epiphyseal cartilage thickness, serum somatomedin activity and liver DNA polymerase: no growth promoting activity was found [4].

Tsai and coworkers [5] reported growth promotion

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of vascular smooth muscle cells by homocysteine (HC), we reported increased growth by homocysteine thiolactone in a clonogenic assay [6], collagen accumulation in aortic tissue in vivo [7] and increased cyclin dependent kinase (CDK) in aortic tissue of rats fed homocysteine but not homocysteic acid [8].

Based upon these observations we decided to study CDK in plasma of patients with hyperhomocyst(e)inemic states and the effect of HC and HCA on CDK in vitro. We could not find any growth promoting effect of homocysteic acid in vitro in a clonogenic assay but detected growth promotion by homocysteine in vitro and in vivo by the correlation with CDK, a key enzyme involved in the starting of mitosis [9].

2. Patients, materials and methods

2.1. Patients

Plasma of 10 patients with hyperhomocysteinemia was obtained from patients treated at the University Hospital Nijmegen. Nine of them had cystathionine β-synthase deficiency and one had the thermolabile form of methylenetetrahydrofolate reductase deficiency [10].

Cystathionine β-synthase deficiency was established by severely elevated levels of homocysteine and methionine and decreased ($<5\%$ of normals) enzyme activity in fibroblasts. These patients were examined for plasma homocyst(e)ine, homocysteic acid and CDK levels. EDTA blood was drawn, put on ice and centrifuged 10 min at $3000\times g$ within 1 h. The characterization of patients and clinical data are given in Table 1. Twenty healthy persons (9 females, 11 males; mean age 34 years, S.D. 12 years) were recruited and served as controls.

Plasma samples of patients and controls were stored at −80°C for a period of at most 3 months, identical sample handling procedures were performed and samples were analyzed accordingly.

2.2. Determination of plasma and cell culture supernatant cyclin dependent p34cdc2 kinase (CDK)

The detection of p34cdc2 kinase (CDK) was achieved by an enzyme linked immunosorbent assay (ELISA) using the commercially available kit In vivo CDK ELISA ([8]; Paracelsian Biotechnology for Toxicological Testing and Research, Ithaca, NY).

The principle of the assay is that proliferative stimulation is determined through the detection of CDK. In the G_0 stage of the cell cycle expression of CDK is minimal but as cells are stimulated into G_1 the expression of CDK increases.

The standards cd RLHCl Positive CDK Control

Table 1
Clinical characterization of patients

Patient	Age		Sex	Height[b] (cm)	Weight[b] (kg)	Treatment				
	at diagnosis[a]	at study				Marfanoid[c]	Vit. B_6	Folate	Vit. B_{12}	Betaine
1B53	25	31	m	188	80	−	+			
2M61	23	33	m	190	89	+	+		+	
3B61	26	33	m	191	77	+	+	+		+
4B58	27	36	m	191	87	+	+	+	+	+
5S25	61	65	f	165[e]	56	+				
6V43[d]	50	50	m	170	64		+	+		
7D67	3	27	m	187	77		+	+		+
8D65	4	28	m	164	84		+	+		+
9U78	7	16	f	171	64		+	+	+	
10G62	31	31	f	180	63	+				

[a]Treatment started since diagnosis except in patient 6.
[b]Height/weight at study.
[c]Marfanold because of dolichostenomelia, arachnodactyly or thorax deformation.
[d]5-Methylenetetrahydrofolate reductase deficiency because of thermolability.
[e]Height in the presence of kyphoscoliosis.

and cd RLLCl Negative CDK Control were used for calibration. The supplier's instructions were followed.

CDK values are expressed in fM/l (plasma or cell culture supernatant).

2.3. Determination of homocyst(e)ine and homocysteic acid

The principle of Fermo and coworkers describing the concomitant determination of total homocysteine (tHcy) and homocysteic acid (HCA [11]) was applied with minor modifications [12].

200 μl of plasma was added to 300 μl distilled water (HPLC grade, Merck), 300 μl of 9 M urea pH 9.0, 30 μl amyl alcohol, 45 μl sodium tetraborate/DMSO solution (3 M $NaBH_4$ in 0.4 N NaOH:DMSO 2:1). This mixture was incubate 30 min at 50°C, then 500 μl of 6% perchloric acid was added, the solution was mixed and spun down. 500 μl of the supernatant were added to 100 μl iodoacetic acid (9.3 mg iodoacetic acid in 0.1 M borate buffer pH 9.5) and 100 μl of 3 M NaOH. 100 μl of this solution was reacted with 100 μl OPA solution (Pierce 26025) and 50 μl was injected after a reaction time of 60 s.

A Waters system controller 600E, a Waters sample 715 processor, a Waters 470 fluorescence detector and the Masima 825 Data processing system were used.

The solvents used for elution were solvent A: water:propionate buffer:acetonitrile (60:30:10); propionate buffer consisted of 15.68 ml propionic acid+49.6 g Na_2HPO_4 anhydride in distilled water, adjusted to pH 6.5. Solvent B was water:acetonitrile:methanol (45:30:25). The gradient was as given previously [12]. Detection was at 230 nm excitation and 417 nm emission. The column was a Beckman Ultrasphere ODS C18, 250×4.6 mm.

As revealed by previous experiments we could rule out that the reductive step used in this method does not affect HCA levels; furthermore, no effect of HCA on tHcy or vice versa was observed. The stability of HCA was preserved at room temperature for 3 days and was unchanged for a period of 3 months at the storage temperature of −80°C (data not shown). The CV of the method listed above was 3.9% for tHcy and 4.1% for HCA; recovery was 96.2%. Samples from cases and controls were analyzed on the same day in the same run.

2.4. The clonogenic assay

The HT 29 human colonic carcinoma cell line was purchased from ATCC (American Type Culture Collection, Rockville, MD, USA).

The HT 29 human colonic carcinoma cell line was grown in RPMI 1640 medium supplemented with 10% heat inactivated FBS (Gibco, Grand Island, NY, USA), penicillin (100 U/ml) and streptomycin (100 U/ml). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air.

For subculture, monolayer cells were dispersed with 0.25% trypsin plus 1 mM EDTA at 37°C for 30 min. After centrifugation the cells were resuspended in fresh medium.

For the clonogenic assay exponentially growing cells were seeded in 24 well plates in medium containing 10% FBS at a density of 150 cells per well. Six hours after seeding various concentrations of the test compounds homocysteine thiolactone (HCTL) and homocysteic acid (HCA) were added at 0–125 μg/ml (see Fig. 1). The cells were then maintained in the medium for 10 days. Triplicate samples were used for each compound concentration. Colonies consisting of more than 50 cells were counted [13].

1×10^6 HT 29 cells in culture flasks were incubated under identical conditions with homocysteine or homocysteic acid in the concentration shown in Fig. 1 and CDK was determined in the supernatants.

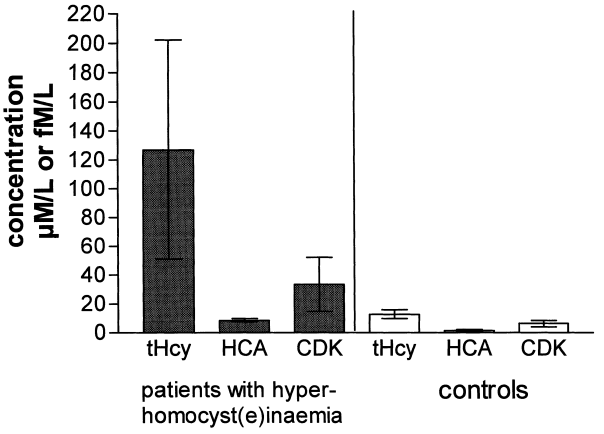

Fig. 1. Presentation of tHcy, HCA and CDK in patients with hyperhomocyst(e)inemia and controls.

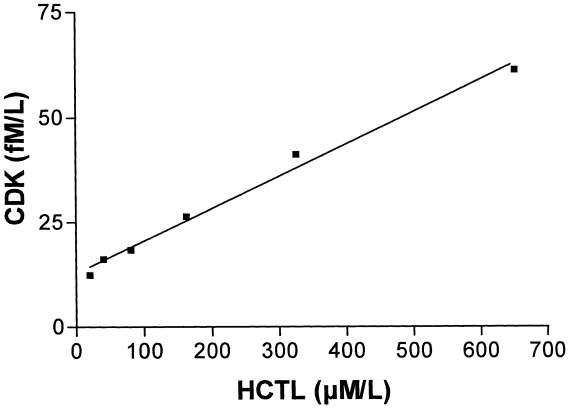

Fig. 2. CDK levels parallelled HCTL concentrations in a clonogenic assay.

2.5. Statistical calculations

The Mann-Whitney U test was applied for the comparison of groups and the Spearman rank correlation was performed for the determination of the correlation coefficient (SAS). Significance was considered at the $P < 0.05$ level.

3. Results

Plasma tHcy and HCA results are given in Fig. 1. Plasma tHcy ad HCA were significantly higher in the group of patients with homocystinuria (Fig. 1). tHcy and HCA levels did not shown any significant correlation.

Plasma CDK levels are presented in Fig. 2. They were significantly elevated in patients with homocystinuria. In the patients group tHcy ($r = 0.95$, Spearman rank correlation) but not HCA levels correlated significantly with plasma CDK levels.

CDK significantly parallelled cell culture supernatant HCTL levels ($r = 0.98$; Fig. 2). HCA did not increase CDK levels in the supernatants (data not shown).

4. Discussion

Plasma tHcy and HCA levels of patients with hyperhomocysteinemia were significantly higher than in the control panel. HCA levels did not, however, correlate with tHcy.

Even the highest plasma Hcy level of 252 µmol/l was not accompanied by a corresponding rise in HCA levels. This does not rule out the possibility that HCA could be responsible for excess growth in homocystinuria. The lack of correlation between tHcy and HCA, however surprising, could be explained by differences in the HC oxidizing enzyme systems as well as by non-enzymatic reactions: the oxidant status, reported to be changed in homocystinuria [14], as well as medication (betaine, vitamins B_6, B_{12}, folate) may interfere with HCA handling or formation. The fact that no correlation of HCA with CDK was found makes the hypothesis that HCA induces excess growth rather improbable. The strong correlation of tHcy with CDK suggests a role of HC in excessive growth.

In 1988 Draetta and coworkers reported on the synthesis and distribution of a 34 kDa protein in rat tissues and cell lines. They found that increased synthesis of p34 was due to an increase in abundance of translatable p34 mRNA. Their data were consistent with the possibility that p34 played a role in cell division in higher vertebrates [15]. Labbe and coworkers reported in the same year that in starfish and amphibian oocytes the activity of a major protein kinase (CDK) increased dramatically at meiotic and mitotic nuclear divisions [16]. Riabowol and coworkers showed one year later that cdc2 CDK was a component of M phase promoting factor in *Xenopus* oocytes and that the homologous kinase in human HeLa cells was maximally active during mitosis [17]. McGowan and coworkers have shown that the activity of that kinase was regulated in a cell cycle dependent manner by reversible phosphorylation and through association with other proteins [18]. A further characterization of cyclin dependent kinases in the human system was given in a recent paper by Lucas and coworkers [19]. Further elucidation of the system was provided by Dutta and Stillman showing that cdc2 family kinases phosphorylate a human cell DNA replicating factor, RPA, and activate DNA replication [20].

We used that enzyme to test the hypothesis that HC itself and not HCA – as suggested in the literature – could be responsible for excess growth. To strengthen this hypothesis we tested HCTL and HCA for their growth promoting activity in a clonogenic assay. Homocysteine as HCTL clearly in-

creased proliferation in contrast to its oxidized form, HCA [6].

A publication in 1956 showed that thiols do play a role in growth promotion: Stern reported in *Science* that thiol groups are important for cell division and referred to the observation that the nitroprusside reaction in proliferating tissues was high in proliferating tissues and that 'thiol poisons' i.e. thiol group blocking agents, inhibit cell division, which was reversible by the addition of glutathione or related thiols. He also clearly described the coincidental increase of thiols with mitosis in the lily *Lilium longiflorum* [21]. Further related information on thiols and cell division was published by Mazia [22].

Our observation that plasma tHcy correlates with a cyclin dependent kinase suggests that HC and not HCA mediates growth in patients with homocystinuria and that this effect could be mediated via cyclin dependent kinases. In a previous paper we have shown that HC increased CDK protein and mRNA steady-state levels [8] but we cannot exclude that homocysteine may work by activating protooncogenes which in turn subsequently would activate CDK. The interaction between protooncogenes and cdk is well-documented and this system in turn could be involved in growth promotion [23–25].

The innovative finding of this report is the association of HC with a growth related kinase which could serve as a possible explanation and a hypothesis together with the findings in the clonogenic assay for excess growth and proliferation in hyperhomocysteinemic states.

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