Prolidase Deficiency in Cultured Human Fibroblasts: Biochemical Pathology and Iminodipeptide-Enhanced Growth

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ABSTRACT. Prolidase deficiency is a rare autosomal recessive disorder characterized by iminodipeptiduria, severe skin ulcers, recurrent infections, and mental retardation. The enzyme prolidase hydrolyzes dipeptides containing C-terminal proline or hydroxyproline. We investigated the metabolic abnormality caused by prolidase deficiency in human cultured skin fibroblasts. These studies were undertaken to test biochemical hypotheses regarding the metabolic origins of the skin lesion occurring in this disease. Our results indicate that prolidase plays a major role in the recycling of dipeptide-bound proline. Control fibroblasts were able to use iminodipeptides in lieu of proline to sustain normal growth, whereas cells homozygous for the prolidase deficiency mutation were not. Proline derived from iminodipeptides diluted incorporation of radiolabeled extracellular proline into cellular protein in normal cells but not in mutant cells. Substitution of a prolidase-free medium for FCS did not affect the growth rate of control cell lines but increased the doubling time of prolidase-deficient cells by 19% (28% in the presence of iminodipeptides). Iminodipeptides added to control and mutant cells maintained in serum-free medium showed no adverse effects on protein synthesis. These results are consistent with a mechanism of biochemical pathology in which proline deprivation caused by the enzyme deficit is a primary cause of damage to skin cells. Prolidase regulation by product and substrate was studied. A 44% decrease in activity was observed in fibroblasts grown for 3 wk in proline-containing medium relative to proline-free medium. However, cells grown in medium in which iminodipeptides replaced proline showed no significant difference in prolidase activity. (*Pediatr Res* 32: 479–482, 1992)

Abbreviations

PD, prolidase deficiency
MEM, minimal essential medium
TCA, trichloroacetic acid
IDP, iminodipeptide
glypro, glycyl-L-proline

Prolidase deficiency (McKusick 26413) is a rare, autosomal recessive disorder characterized by iminodipeptiduria, skin lesions, recurrent infections, and mental retardation (1). The enzyme prolidase (EC 3.4.13.9) is a homodimer with a subunit

molecular mass of 54.3 kD (2) and a requirement for Mn^{2+} ions. Prolidase is ubiquitously distributed and catalyzes the hydrolysis of C-terminal proline and hydroxyproline-containing dipeptides. The enzyme presumably functions in the degradation of both dietary protein and in the turnover of body protein and is particularly important in collagen catabolism. The gene, PEPD, is encoded on chromosome 19p13.2 (2). The relationship between the enzymatic and clinical phenotype is unknown, and no form of treatment is presently available for patients with this condition.

We have studied the biochemical pathology of PD using the cultured human skin fibroblast as a model for investigation of the metabolic role of prolidase in skin cells. The principal difficulty in the cultured cell model is the routine use of FCS in the culture medium. Serum contains prolidase, which may prevent or "cure" the expression of the defect in the cultured cell. We have therefore used a serum substitute that is prolidase-free to develop a selective medium in which optimal growth of the cell is dependent upon the expression of the normal allele at the PEPD locus.

We describe the effects on normal and prolidase-deficient mutant cells of 1) growth of cells in medium containing prolidase-free serum substitute, 2) protein synthesis by confluent cells in serum-free medium, and 3) growth of cells in proline-free medium. Our results support the hypothesis that the function of prolidase is reclamation of proline bound in dipeptidyl linkage for cellular protein synthesis.

MATERIALS AND METHODS

Prolidase assay. The procedure of Myara *et al.* (3) was used with the following modifications: enzyme solutions were preincubated with 1 mM $MnCl_2$ overnight to maximize prolidase activity. Glypro (Sigma Chemical Co., St. Louis, MO) was used at a final concentration of 50 mM. Proline released by dipeptide hydrolysis was determined by the method of Chinard (4).

Tissue culture. Human fibroblasts were grown in T75 (or T175) culture flasks (Falcon, Mississauga, Ontario, Canada) containing Eagle's MEM (GIBCO, Burlington, Ontario, Canada) supplemented with 10% FCS, 1% Ultraser (GIBCO), 1% ITS, or ITS+ (Collaborative Research, Bedford, MA) serum substitutes. The latter two preparations contain bovine insulin, Fe-saturated transferrin, and the sodium salt of selenous acid. ITS+ contains, in addition, BSA and linoleic acid. For growth experiments, two different MEM products were used. MEM₁ (GIBCO preparation 410–1500) is the more complete medium, containing aspartate, asparagine, glutamate, serine, and proline, whereas MEM₂ (GIBCO preparation 410–1100) is deficient in these amino acids. In addition, MEM₂, but not MEM₁, contains choline bitartrate. Cells were grown at 37°C in 5% CO₂ and were fed every 3 or 4 d.

The normal fibroblast cell lines, MCH 39 and MCH 70, were

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derived from foreskin explants after circumcision. Prolidase-deficient cell lines were obtained from a 27-y-old clinically affected female (WG 1625) and a male infant (WG 1077) detected by neonatal screening (5) who remains asymptomatic at the age of 8 y. Both individuals had massive iminodipeptiduria. Determination of prolidase sp act in these cell lines are reported in Table 1.

Growth curves. Cell counting was performed after trypsinization using a Coulter counter (Coulter Electronics, Burlington, Ontario, Canada). All reported cell numbers represent a mean of three Petri dishes, each counted in triplicate. Doubling times calculated are during the early log phase of growth.

Protein synthesis. Fibroblasts were grown to confluence in P60 dishes, the medium was removed, and the cell monolayers were washed twice with isotonic PBS. Serum-free medium was then added along with 10 μ Ci of the radiolabeled amino acid (L-2,3- 3 H-proline, 52.0 Ci/mmol; L-4,5- 3 H-leucine, 60.0 Ci/mmol; New England Nuclear, Boston, MA). After the labeling period, the medium was removed the cells washed twice with PBS and lysed with a solution of 2.5% Triton X-100 and 3 M NaCl.

The radioactivity in the TCA-precipitable component of the cell lysate was determined by the method of Mans and Novelli (6). The protein concentration of the lysate was determined by the method of Lowry *et al.* (7) using crystalline BSA (Sigma) as protein standard.

Uptake of radiolabeled hydroxyproline by fibroblasts. The same procedure as above was employed using hydroxyproline (4-OH-(G)- 3 H-proline, 5.5 Ci/mmol; New England Nuclear) except that the radioactivity of the total cell lysate itself was determined by scintillation counting without TCA precipitation.

RESULTS

Commercially available serum substitutes were screened to identify a prolidase-free source of cellular growth and attachment factors. Table 1 records the prolidase activity of FCS and three serum substitutes.

Growth rates of control and PD cells were compared in FCS and ITS or ITS+ containing MEM₁. The log-phase doubling times for these growth curves are presented in Table 2. ITS medium significantly increased the doubling time for control cells relative to FCS-containing media, whereas ITS+ medium did not affect the growth characteristics of control cells but did selectively decrease growth of the prolidase-deficient cell lines. These results suggested two hypotheses: 1) IDP substrates accumulating in the absence of prolidase inhibit cell growth or 2) the activity of prolidase is required for optimal cell growth by virtue of its role in reclamation of proline (or other amino acids). Alternatively, the differential response to ITS+ medium may be related to genetic differences between the cell lines other than those occurring at the PEPD locus. The two PD cell lines were used to test the putative antimetabolite activity of IDP substrates

Table 1. Prolidase activity of FCS, serum substitutes, and human fibroblast cell lines

Serum or substitute	Prolidase activity (μ mol glypro hydrolyzed/mL/h)
	(μ mol glypro hydrolyzed/mg protein/h)
FCS	8.9
Ultraser G	47.4
ITS	<0.1
ITS+	<0.1
Cell lines	
MCH 39	39.7
MCH 70	53.4
WG 1077	<0.1
WG 1625	<0.1

Table 2. Effect of IDP on growth of fibroblasts

Cell line	Medium used	Doubling time (h)*
MCH 39	MEM ₁ + 10% FCS	55.5 $\pm$ 4.3
MCH 39	MEM ₁ + ITS	66.4 $\pm$ 5.9
MCH 39	MEM ₁ + ITS+	57.0 $\pm$ 4.0
MCH 39	MEM ₁ + ITS+, + 4 IDP†	54.3 $\pm$ 5.1
WG 1625	MEM ₁ + 10% FCS	85.8 $\pm$ 6.8
WG 1625	MEM ₁ + ITS+	102.1 $\pm$ 7.3
WG 1625	MEM ₁ + ITS+, + 4 IDP†	109.6 $\pm$ 8.2

* Growth curves were performed with three dishes for each time point, and three samples from each dish were counted.

† Five mM each of glycyl-L-proline, leucyl-L-proline, phenylalanine-L-proline, and alanyl-L-proline.

Table 3. Effect of glycl-L-proline on incorporation of leucine and proline into protein

Cell line	Medium	Leucine incorporated (cpm/ μ g)	Proline incorporated (cpm/ μ g)
MCH 39	MEM ₁	13 586 $\pm$ 687	4 129 $\pm$ 422
MCH 39	MEM ₁ + 5 mM glypro	12 905 $\pm$ 579	2 069 $\pm$ 196
MCH 70	MEM ₁	8 720 $\pm$ 594	2 338 $\pm$ 303
MCH 70	MEM ₁ + 5 mM glypro	8 557 $\pm$ 1 316	1 725 $\pm$ 242
WG 1625	MEM ₁	9 298 $\pm$ 1 191	1 063 $\pm$ 31
WG 1625	MEM ₁ + 5 mM glypro	10 301 $\pm$ 1 137	1 101 $\pm$ 186
WG 1077	MEM ₁	5 935 $\pm$ 160	3 239 $\pm$ 470
WG 1077	MEM ₁ + 5 mM glypro	6 328 $\pm$ 243	3 438 $\pm$ 253

in a culture system in which neither cells nor medium possessed the enzymatic activity required for the hydrolysis of these compounds. As seen in Table 2, the combination of the four IDP did not retard the growth of the control cells but had a small effect on the PD cell lines growing in prolidase-free ITS+ medium.

The effect of IDP on protein synthesis by confluent cells in serum-free medium as determined by following incorporation of both 3 H-leucine and 3 H-proline into TCA-precipitable material is shown in Table 3. In none of the cell lines tested did glypro alone or the combination of four IDP (data not shown) decrease the rate of incorporation of leucine into protein, indicating that these dipeptides do not cause inhibition of protein synthesis. The reduction of proline incorporation in the presence of glypro, however, occurred only in the two normal cell lines, although not to the same extent.

The absence of glypro inhibition of leucine incorporation indicates that reduction of proline incorporation cannot be due to protein synthesis inhibition. An alternative site of IDP inhibition of proline incorporation might be the interaction of the IDP substrates and proline at a plasma membrane transport carrier. Inhibition of imino acid transport by IDP was ruled out by measuring the effect of these compounds on the uptake of 4-hydroxyproline. This amino acid was used as a transport substrate in lieu of L-proline because the two compounds share a membrane transport carrier (8) but hydroxyproline is not incorporated into protein. These results are shown in Figure 1. The most likely explanation for the observed decrease in proline incorporation by normal fibroblasts in the presence of IDP is the hydrolysis of the IDP by intracellular prolidase and the subsequent dilution of the isotope by the proline produced during hydrolysis.

An alternative hypothesis regarding the role of prolidase is that it functions as a "salvage" enzyme to recover dipeptide-bound proline and make it available for protein synthesis. Experiments were performed to determine whether 1) proline in the growth medium is limiting for growth of normal fibroblasts, 2) the activity of prolidase in normal fibroblasts is sufficient to supply the proline requirements of a proline-starved cell, and 3) proli-

Fig. 1. Uptake of 4-hydroxy-(G)-³H-proline in fibroblasts in the presence (■) and absence (▨) of glycyl-L-proline.

Table 4. IDP-dependent growth

Medium	Doubling times (h)*			
	MCH 39	MCH 70	WG 1625	WG 1077
MEM ₂	80.2 ± 4.1	103.4 ± 9.3	77.8 ± 4.7	109.6 ± 8.7
MEM ₂ + Pro 1 mM	45.8 ± 3.7	81.3 ± 8.1	38.9 ± 2.9	85.2 ± 7.6
MEM ₂ + glypro 10 mM	47.0 ± 5.2	85.6 ± 6.8	74.2 ± 5.5	113.8 ± 9.3
MEM ₂ + Pro 1 mM + glypro 10 mM	49.0 ± 4.9	82.3 ± 7.9	34.3 ± 3.5	82.9 ± 7.1

* Growth curves were performed with three dishes for each time point, and three samples from each dish were counted.

dase-deficient cell lines are unable to satisfy proline requirements from IDP precursors.

Table 4 shows the results (expressed as the log-phase doubling times, in h) of a growth experiment where both mutant and normal fibroblasts were grown in proline-deficient MEM₂ (see Materials and Methods for description) and in MEM₂ to which proline has been added. Doubling times for cell lines in unsupplemented MEM₂ are high, indicating slow growth. A comparison of MCH 39 doubling times in MEM₂ (Table 4) can be made with the doubling time in the more complete MEM₁ (Table 2). Although MEM₂ differs from MEM₁ by the absence of five amino acids from the former preparation, it is evident from Table 4 that supplementation of MEM₂-grown cultures with proline alone can increase the growth by as much as 2-fold and that the growth rate increase occurs in both control and prolidase-deficient fibroblasts. The 25% higher growth rate of control cells in MEM₂ plus 1 mM proline suggests that proline concentration (0.1 mM) in MEM₁ is limiting for cell growth.

The ability to sustain optimal growth when glypro replaces proline, however, is limited to normal genotypes and does not occur in the two prolidase-deficient cell lines. Table 4 shows the doubling times of the four cell lines when both proline and glypro are present in the medium. In this experiment, the doubling times are identical to those in which proline is the sole supplement. This experiment shows that the inability of prolidase-deficient cells to grow optimally in the presence of glypro is due to inability to convert the substrate to proline, not to an inhibition of growth by unhydrolyzed prolidase substrates.

To determine whether prolidase activity in fibroblasts is regulated by product and/or substrate, normal fibroblasts were grown for 21 d in media containing proline, glypro, and a combination of both. The sp act of cells grown in each condition are presented in Table 5. A control group had no proline or glypro added to the growth medium but contained 0.1 mM proline present in MEM₁.

The only deviation from the control value occurs when there

Table 5. Regulation of fibroblast prolidase activity

Medium	Prolidase activity (μmol/mg/h)	%
MEM ₁ - Pro	56.7 ± 10.2	100.0
MEM ₁ + Pro 1 mM	31.8 ± 7.6	56.1
MEM ₁ + Glypro 5 mM	58.6 ± 8.1	103.4
MEM ₁ + Glypro 5 mM + Pro 1 mM	53.9 ± 13.6	95.1

is an excess of proline (1 mM) in the growth medium causing a decrease in sp act of 50%, suggesting some end product regulation of prolidase. The addition of IDP alone does not enhance prolidase activity. In the presence of proline and substrate, however, no decrease in activity is observed. The results suggest that, at normal MEM₁ proline concentration, cell growth depends upon maximal expression of the enzyme.

DISCUSSION

PD is both selective in its site of clinical expression and variable in its severity. Six of 31 patients reported are asymptomatic (1) and two of these are older siblings of severely affected patients (9, 10). Because the skin is the most frequently affected tissue clinically, we believed it would be useful to determine whether one of the more easily cultured skin cell types, the fibroblast, would manifest any growth or metabolic abnormality in culture that could be attributed to the enzyme deficiency. A cellular phenotype whose appearance could be subjected to experimental manipulation would be of benefit in the investigation of such questions as: What is the biochemical pathology responsible for the clinical phenotype? What is the biochemical basis for the variable clinical expression both within and between families? Can any experimental evidence be obtained in support of dietary manipulation as a therapeutic strategy for PD?

Previous metabolic studies of PD have used the fibroblast as a model, but the presence of prolidase in the FCS component of growth medium has been ignored (11). Thus, PD cells grown in FCS-containing medium may have their phenotype corrected by extracellular prolidase. This would occur if the excretion of IDP into the medium is a critical event in the development of the cellular phenotype. In the cultured fibroblast, IDP that are produced during protein catabolism are freely diffusible across the plasma membrane. In prolidase-containing medium, the amino acid products of dipeptide hydrolysis can be recycled back to the cell.

The demonstration that proline, which is not an essential amino acid, is limiting for the optimal growth of fibroblasts was an important requirement for clarifying the role of prolidase. The finding that normal fibroblasts are able to use glypro to satisfy their proline requirement demonstrates that prolidase functions *in vivo* to recycle proline and that the clinical phenotype in PD is likely to be related to a failure to recycle this amino acid. This interpretation is consistent with previous studies showing that a substantial portion of proline incorporated into collagen in rat skin fibroblasts is recycled (12) and that approximately 10% of newly synthesized cultured fibroblast protein is rapidly degraded even during log-phase growth (13).

This conclusion is not supported, however, by clinical experience with PD patients. In three studies patients did not respond to oral supplementation with proline (14–16), whereas in a fourth study in which patients were reported to have improved clinically the results were not definitive because of the presence of other dietary supplements such as Mn and large doses of vitamin C (17). It has also been reported that serum levels of proline are within normal limits in PD patients (18).

This study demonstrates that IDP do not act as antimetabolites in fibroblasts. This does not rule out action against another cell type; furthermore, the IDP tested included only four of the most abundantly excreted compounds and not the entire group of 19

possible IDP. A potential investigative tool that may be useful in clarifying the mechanism underlying the skin phenotype in PD is the pharmacologic agent captopril (D-3-mercapto-2-methylpropanoyl-L-proline) (Merck, Montreal, Canada). Captopril is a common antihypertensive drug that is a structural analog of the IDP cysteinylproline and alanylproline. It is believed to exert antihypertensive effects by competitively inhibiting the carboxypeptidase, angiotensin-converting enzyme (19). This drug also competitively inhibits prolydase *in vitro* and *in vivo* (20).

Some individuals using captopril may suffer skin lesions (19) that bear a resemblance to those presenting in prolydase-deficient individuals. This response can be corrected by a reduction in dosage. The mechanism underlying the captopril-induced skin lesions is not well understood, but Wilkin *et al.* (21) proposed that the lesions are inflammatory responses elicited by the persistence of kinins resulting from the inhibition of kininase II (also a carboxypeptidase) by captopril.

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