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Pseudo arylsulfatase A deficiency

Biosynthesis of an abnormal arylsulfatase A

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Pseudo arylsulfatase A deficiency, an asymptomatic condition, and metachromatic leukodystrophy, a severe neurodegenerative disease, are both associated with profound reductions of arylsulfatase A activity in man. We now report that with metabolic labelling, cultured pseudo deficient cells synthesized about 20% of the normal amount of arylsulfatase A at a reduced rate of apparent synthesis and increased rate of degradation. However, in the presence of ammonium chloride which stimulated secretion of lysosomal enzymes, these cells synthesized about 80% of the normal amount of enzyme protein. Hence, the defect in pseudo arylsulfatase A deficiency is associated with labile arylsulfatase A molecules which can be stabilized if they are diverted from intracellular storage.

Lysosomal enzyme; Ammonium chloride; Lipidosis; Metachromatic leukodystrophy

1. INTRODUCTION

Pseudo arylsulfatase A deficiency (PD) and metachromatic leukodystrophy (MLD) are autosomal-recessive conditions in man associated with profound reduction in the activity of the lysosomal enzyme arylsulfatase A (ARA, EC 3.1.6.1) [1–3]. Although both conditions are associated with allelic mutations of the same gene coding for ARA [4], clinically, MLD presents as a severe neurodegenerative disorder whereas PD is a benign condition. Cultured fibroblasts from MLD patients either did not produce cross-reacting-material to ARA [5] or ARA that was normal in size but highly unstable [6]. Those from PD patients produced ARA that was reduced in quantity and in size by about 3 kDa when compared to normal [5,6]. We now report abnormalities in the biosynthesis of ARA by PD cells and how they may be partially reversed under appropriate experimental conditions.

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2. MATERIALS AND METHODS

2.1. *Tissue culture*

Fibroblasts from 3 normal controls (Ag, A25, Sellers) were established from skin biopsies and those from patients with PD were supplied by Dr H. Kihara, UCLA (578) and Dr E. Kolodny, Eunice Kennedy Shriver Center for Mental Retardation, Inc. (4835, 3475). They were maintained under the usual culture conditions [4]. For metabolic labelling, each 25 cm^2 flask was seeded with 1×10^5 cells. Media were changed once a week. After 15–18 days the cells were incubated for 1 h in leucine-free Waymouth medium MAB 87/3 (KC Biological, KS) and then pulsed with 200 μCi per flask of [4,5-^{3}H]leucine (NEN, Lachine, Quebec) in 3 ml Waymouth medium. For chase experiments, the radioactive medium was

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removed after the pulse, and the cells washed twice with 5 ml medium before incubation with Waymouth medium containing 5 μg/ml of leucine for the chase periods. In experiments with ammonium chloride, the medium was supplemented with 10 mM NH_4Cl [7].

2.2. *Preparation of cell extract and medium*

The cells in each flask were washed twice with 5 ml phosphate-buffered saline and extracted with 500 μl of 1% Nonidet P-40 (Sigma, St. Louis) in Tris-buffered saline, pH 7.4. Protein was determined according to Bradford [21]. 25 μl leupeptin (2 mg/ml, Boehringer Mannheim, Quebec) were added to the extract. From the culture medium, protein was precipitated with 75% saturation of ammonium sulfate [7], dissolved in 200 μl of 0.01 M Tris-HCl, pH 7.4, containing 0.15 M NaCl and 0.02% NaN_3, desalted with Sephadex G-50 (Pharmacia, Dorval, Quebec) and leupeptin was added as for the cell extract.

2.3. *Immunoprecipitation, electrophoresis and fluorography*

The immunoprecipitation [6], solubilization [8] and electrophoresis [9] were performed as described. The anti-ARA antiserum was kindly supplied by Drs G. Bach and E. Neufeld [6]. The radioactive bands were detected by fluorography and quantitated with a Bio-Rad 620 densitometer and 3392A integrator (Bio-Rad, Mississauga, Ontario). Each band was scanned three times and the mean area under the peak was related to radioactivity standards incorporated into each

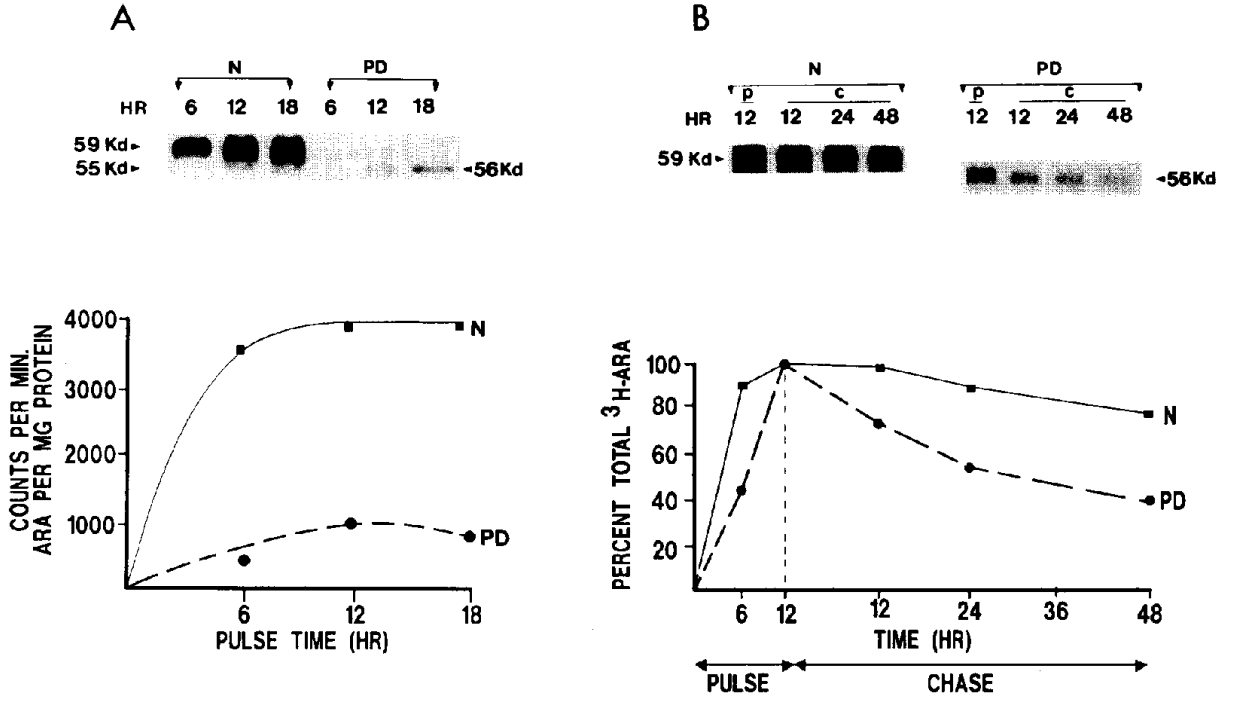

Fig.1. (A) Synthesis of ARA by normal (Ag) and PD (578) fibroblasts. Cells were pulsed with 200 μCi [^{3}H]leucine per flask. Following extraction with Tris-buffered saline, pH 7.4, containing 1% Nonidet P-40, fibronectin was immunoprecipitated with 5 μl rabbit anti-human fibronectin antiserum. ARA was isolated by immunoprecipitation with 2 μl goat anti-ARA antiserum overnight at 4°C. Immunoprecipitates were analyzed by SDS-polyacrylamide electrophoresis on 10% gels at 35 mA for 300 min. The radioactive peptide was quantified by densitometry. Each point is the mean of 4 independent experiments with SD of less than 10%. (B) Degradation of ARA in normal and PD fibroblasts. Following a 12 h pulse, fibroblasts were chased for 12–48 h. ARA levels were estimated as above. Each point is the mean of 3 independent experiments with SD of less than 10%. Molecular mass was estimated by comparison with radioactive standards.

fluorograph. The linear range of response between radioactivity and area under the peak was from 250 to 1910 cpm.

3. RESULTS

The results reported here were obtained for the normal cell line, Ag and PD the cell line, 578. Similar results (not shown) were obtained with A25 and Sellers (normal) and 4835 and 3475 (PD).

The biosynthesis of ARA was followed by pulse-chase experiments as shown in fig.1. PD and normal cells showed maximal incorporation of radioactivity into ARA by 12 h. However, their apparent initial rates of synthesis were drastically different. For example, after pulsing for 6 h, PD cells achieved only 50% of maximal labelling while normal cells had incorporated 90% of the maximal radioactivity already. In addition, after 12 h of pulse, the level of labelled ARA was about 4000 cpm/mg protein for normal fibroblasts, while that for PD fibroblasts was only about 1000 cpm/mg (fig.1A). During the course of this labelling, normal cells synthesized a major ARA species of 59 kDa and a very minor one of 54–55 kDa while PD cells synthesized only a single detectable species of ARA of 56 kDa.

Chase experiments demonstrated that the apparent rates of degradation of ARA between normal and PD cells were also markedly different (fig.1B). A 12 h pulse followed with a 12 h chase showed no loss of radioactive ARA in normal cells while 30% of the ARA in the PD cell had already been degraded. The apparent half-life of the radioactive ARA was estimated to be 33 h in the PD cells whereas in normal cells only 12% of the labelled ARA was degraded in the same period. Neither cell line showed any change in molecular mass of ARA subunits during the chase period.

When the biosynthesis of ARA was followed with pulse experiments but in the presence of NH_4Cl, the PD cells demonstrated an unexpectedly high level of synthesis when compared to the normal (fig.2). The apparent rates of synthesis of the intracellular ARA in both normal and PD cells were linear in the first 6 h of pulse. The PD cells accumulate about 600 cpm/mg protein compared to about 1200 cpm/mg protein in normal cells. By 12 h of pulsing, the ARA in PD cells had again achieved maximal incorporation in the cell at about 850 cpm/mg protein, compared to about 1700 cpm/mg protein in the normal cells (fig.2A). This represented 50% of normal level instead of the 25% in the absence of NH_4Cl.

When the secreted form of ARA was followed in PD cells, the level of biosynthesis in the presence of NH_4Cl was even more elevated, surpassing that of the normal (fig.2B). In normal cells, secreted ARA reached the maximal plateau of incorporation at 1700 cpm/mg protein from 9 to 18 h of pulse. In PD cells, incorporation continued to increase and approached the maximum only by 15 h of pulsing. Even at 12 h of pulsing, the level of radioactivity of ARA in the PD media reached about 2000 cpm/mg protein, surpassing that of the normal by about 20%. If NH_4Cl was omitted from the media, neither cell type secreted detectable levels of labelled ARA (not shown).

With normal cells, the presence or absence of NH_4Cl in the medium did not alter the molecular masses of either the intracellular or secreted ARA subunits (figs 1A, 2A or 2B). However, with PD cells, in the presence of NH_4Cl, both the intracellular and secreted ARA subunits were slightly larger, i.e. 57 kDa in fig.2, compared to 56 kDa in fig.1.

4. DISCUSSION

The present findings showed that the low level of ARA in PD fibroblasts observed previously [5,6] was the result of increased degradation. The apparent initial rate of synthesis of ARA was also reduced by 10-fold in PD fibroblasts, i.e. 60 cpm ARA/mg protein per h as compared with 600 cpm ARA/mg protein per h in normal fibroblasts (fig.1A) or by 3-fold if NH_4Cl, an amine that increases intralysosomal pH and stimulates secretion of lysosomal enzymes [10], was present (fig.2). However, the earliest pulse time was 4 h (fig.2) when the newly synthesized ARA may have been modified or partially degraded already during its passage through prelysosomal compartments. Therefore, the reduction in the apparent rate of synthesis may not reflect the true reduction in the initial rate of synthesis. However, it is very clear that the ARA in PD cells was considerably more labile, with an apparent half-life of one-ninth that of normal enzyme as calculated from fig.1B and unpublished data on chase up to 96 h.

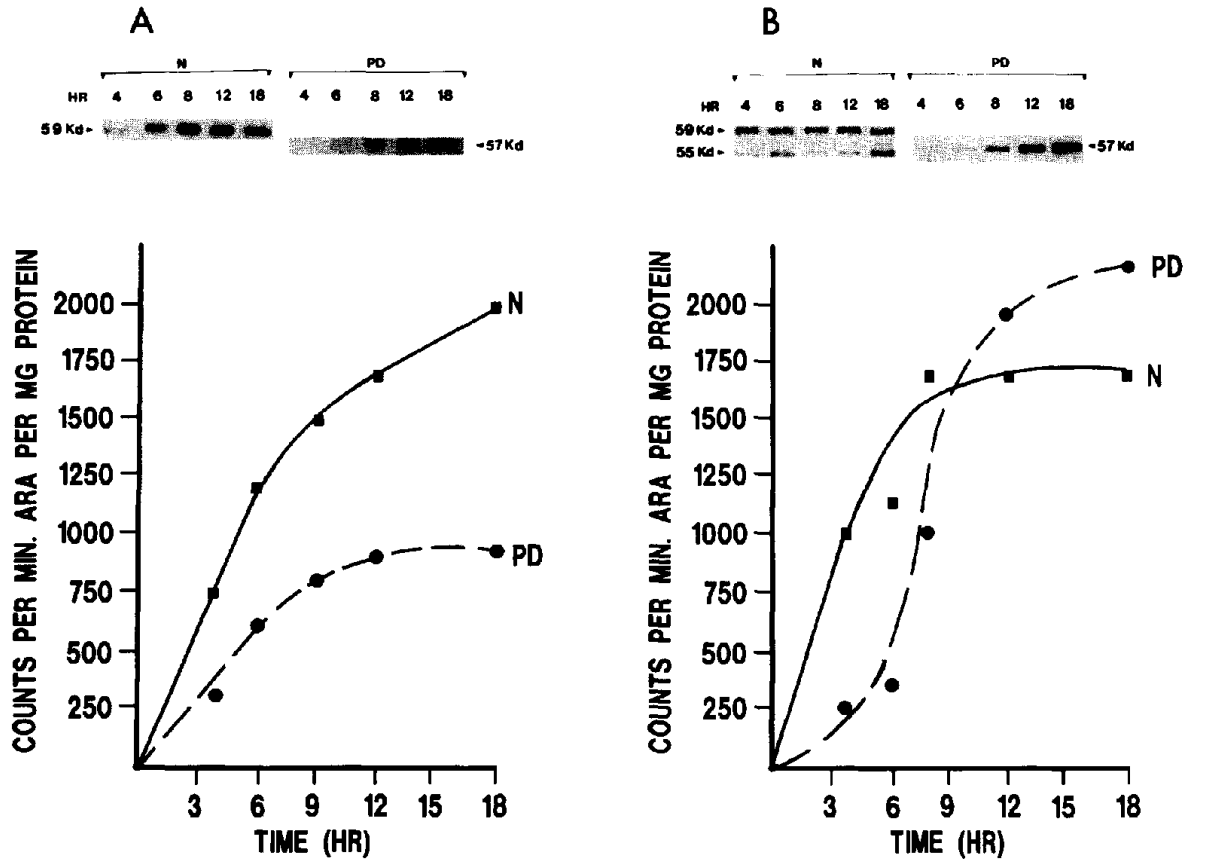

Fig.2. Effect of NH_4Cl on synthesis and secretion of ARA in normal (Ag) and PD (578) fibroblasts. Experiments were performed as described for fig.1 except that the pulse medium was supplemented with NH_4Cl (10 mM). The pulse medium was removed and treated as outlined in section 2. (A) Intracellular ARA was determined as described for fig.1A. Each point is the mean of 3 independent experiments with SD of less than 10%. (B) The media were concentrated with $(NH_4)_2SO_4$, desalted and immunoprecipitated as described above. Electrophoresis was for 330 min at 35 mA. Each point is the mean of 3 independent experiments with SD of less than 10%.

Residual amounts of ARA protein with increased lability were also observed among some MLD cells [11]. This raised the question of why the two forms of ARA deficiencies result in such different clinical manifestations, particularly when previous complementation studies showed that PD and MLD were due to allelic mutations of the same structural gene for ARA [4]. A possible explanation is the different degrees of instability in the two mutants. When the newly synthesized ARA in PD cells was diverted to the secretory pathway by NH_4Cl, and hence protected from the rapid intracellular degradation (fig.1B), the extracellular pool of ARA rose to above normal levels (fig.2B). In contrast, under similar conditions, MLD cells accumulated only 15–46% of the normal extracellular level of ARA molecules [11]. In addition to the relatively greater stability of the mutant ARA molecules in PD, the 5–10% of residual ARA activity in PD cells demonstrated catalytic activity towards both artificial [12] and natural substrates for ARA [13]. In contrast, the residual activity in MLD cells did not possess similar activity. Therefore, the increased stability and authentic ARA activity in PD may have contributed towards the dramatic difference in the clinical outcomes of the two conditions.

Similar pseudo-deficiency conditions for several

lysosomal storage diseases have been reported [14–17]. It is of concern that some of these patients may eventually develop mild lower neuron dysfunction [18,19]. The possibility of similar neurological dysfunction developing in the PD patients cannot be ruled out. The use of protease inhibitors to elevate ARA levels in fibroblasts has produced varying results. Herz and Bach [20] found no increase in ARA enzyme activity in PD cells, whereas Von Figura et al. [11] showed partial restoration of ARA enzyme protein and activity in MLD cells when incubated with thiol proteinase inhibitors. Thus, the therapeutic use of protease inhibitors to increase ARA levels in PD or other lysosomal enzymes in their respective pseudo deficiencies should be further investigated.

In conclusion, our results demonstrate that in cultured fibroblasts from PD patients, the reduced ARA activity is associated with a labile ARA protein which showed an increased rate of degradation in the lysosomes. However, near normal levels of ARA can be restored in vitro by diverting the enzyme from intracellular storage with NH_4Cl-induced secretion.

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