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Non-rhizomelic and rhizomelic chondrodysplasia punctata within a single complementation group

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Abstract

Several patients have been described recently who suffer from a non-rhizomelic type of chondrodysplasia punctata (CDP), but who show all the biochemical abnormalities characteristic of the rhizomelic form of chondrodysplasia punctata (RCDP), a peroxisomal disorder. We have used protease protection experiments and microinjection of reporter-protein-encoding expression plasmids to show that peroxisomal thiolase fails to be imported into peroxisomes in cells from non-rhizomelic CDP patients, as has already been found in cells from classical RCDP patients. Furthermore, complementation analysis after somatic cell fusion indicates that the non-rhizomelic CDP patients are impaired in the same gene as classical RCDP patients. We conclude that defects in a single gene can give rise to both clinical phenotypes.

Keywords: 3-ketosacyl-CoA thiolase; Disorder; Peroxisome; Rhizomelic form of chondrodysplasia punctata; Fibroblast

1. Introduction

The rhizomelic form of chondrodysplasia punctata (RCDP) is an autosomal recessive disease characterised clinically by symmetrical shortening of the proximal extremities, congenital contractures, typical craniofacial dysmorphism with characteristic ocular involvement, severe mental and growth retardation and typical radiological abnormalities [1,2]. RCDP was assigned to the peroxisomal disease category after finding an impaired plasmalogens (ether phospholipid) synthesis [3]. The biochemical abnormalities in RCDP are now known to include (i) a (partial) deficiency of dihydroxyacetonephosphate acyltransferase; (ii) a deficiency of alkylidihydroxyacetone phosphate synthase; (iii) an elevation of phytic acid due to the deficient α-oxidation of phytanic acid and (iv) the presence of peroxisomal thiolase in precursor form [2–6].

These deficiencies are likely to be the indirect result of a defect in a single gene, the protein product of which may be involved in the import of these enzymes into peroxisomes.

Although the above-mentioned subset of peroxisomal functions is deficient in RCDP, peroxisomes in RCDP fibroblasts have been shown to be morphologically normal and capable of importing catalase [7] and peroxisomal proteins containing the (C-terminal) peroxisome targeting signal type 1 (PTS1) [8]. However, proteins containing the type 2 peroxisome targeting signal (PTS2) (present in the presequence of peroxisomal thiolase) are not imported into peroxisomes in RCDP cells [8].

Several patients have been described recently who show chondrodysplasia punctata without the rhizomelic dwarfism (non-rhizomelic CDP), but biochemically show all the peroxisomal deficiencies characteristic of RCDP [9–12]. It was therefore of interest to examine the genetic relationship between the non-rhizomelic CDP patients and the classical RCDP patients which we have earlier found to fall into a single large complementation group [13]. We have done this using complementation analysis following...
somatic cell fusion. In this paper, we show that the four non-rhizomelic CDP patients all belong to the same complementation group as the classical RCDP patient. This complementation group contains, in addition to the four non-rhizomelic CDP patients described in this paper, fibroblasts from nine classical RCDP patients [13].

Fig. 1. Subcellular localisation of the reporter proteins luciferase (A,B) and pyruvate-CAT (C,D) in control (A,C) and CDP1 (B,D) cells. Subcellular localisation of endogenous thiolase in control (E) and CDP1 cells (F).
2. Materials and methods

2.1. Cell culture

Primary human skin fibroblasts were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s Medium and Ham’s F10 (Gibco, Glasgow, UK) supplemented with 10% foetal calf serum (Gibco) under 5% CO₂. The fibroblast lines used in the analysis were from a control subject (85AD5035F), from an RCDP patient (cell line MCI-IE 85AD [13], from a Zellweger Syndrome patient (GOM85AD) belonging to complementation group III according to the Amsterdam nomenclature [15] and from three non-rhizomelic CDP patients (CDP1-4) described in the literature [9-12] and one unpublished patient (CDP4) (Barth).

2.2. Microinjection

24 h before microinjection the cells were plated onto microinjection grids. The needles were made using a PB-7 micropipette puller (Narishage Co., Tokyo) and DNA was injected in reverse PBS (4 mM Na₂HPO₄/1 mM KH₂PO₄/140 mM KCl (pH 7.3)) at a concentration of approx. 0.1 mg/ml. 18 h after microinjection, the cells were processed for immunofluorescence microscopy as previously described [7]. The expression plasmid encoding the PTS1 reporter protein firefly luciferase has been described elsewhere [8]. The expression plasmid encoding the PTS2 reporter protein, comprising the first 15 amino acids of rat thiolase fused onto bacterial chloramphenicol acetyltransferase (pRSV-FLT3-CAT), was a kind gift from S. Subramani (University of California, San Diego, CA) and is described by Swinkels et al. [14].

2.3. Cell fusion and assessment of complementation

Fusion mediated by polyethylene glycol was carried out exactly as described [15]. The fusion efficiency, defined as the percentage of total nuclei in multinucleate cells, was always in the range 60–90%. The cells were processed for immunofluorescence using a monoclonal anti-thiolase antibody 18 h after fusion. The appearance of particulate thiolase using indirect immunofluorescence was taken to indicate complementation.

2.4. Proteinase K protection

Cells were harvested by trypsinisation, washed twice in cold PBS and resuspended to a protein concentration of approx. 1 mg/ml in isotonic sucrose buffer (250 mM sucrose, 20 mM 3-(4-morpholino)propane sulfonic acid (MOPS)) (pH 7.4) containing 125 μg/ml digitonin. The cells were then incubated for 5 min at 37°C in digitonin permeabilisation buffer. This concentration of digitonin causes rupture of the plasma membrane, whereas the peroxisomal membrane remains intact. The protease protection assay (as described in [8]) was carried out on ice using a Proteinase K concentration of 40 μg/ml in the presence or absence of 0.5% Triton X-100. Samples were removed after 0.5 and 1 h, and Proteinase K action was stopped by addition of PMSF to 10 mM. Protection of thiolase was assessed by immunoblot analysis.

2.5. Immunoblot analysis

Samples were subjected to electrophoresis on 10% polyacrylamide gels according to the method of Laemmli. Immunoblot analyses were performed as described previously with the following modifications. Non-specific bind-

| Proteinase K | - | + | + | - |
| Triton X-100 | - | - | + | + |
| Time (min) | 30 60 | 30 60 | 30 60 | 30 60 | 30 60 |

Fig. 2. Accessibility of endogenous precursor thiolase to digestion by proteinase K in permeabilised cells from a neonatal adrenoleukodystrophy (NALD) patient (upper panel) and CDP1 cells (lower panel). C, lysate from control cells, showing 41 kDa mature thiolase. Arrowhead shows position at which 44 kDa precursor thiolase migrates. Band migrating above precursor thiolase is a cross-reacting band unrelated to thiolase.
ing sites were blocked overnight with 10% foetal calf serum in TBS (10 mM Tris (pH 8)/150 mM NaCl) containing 0.2% Tween 20. The primary (polyclonal anti-thiolase [16] and secondary (goat anti-rabbit-conjugated horseradish peroxidase (BioRad)) antibody incubations were performed in TBS containing 4% foetal calf serum and 0.1% Tween 20. The blots were washed extensively after each incubation in TBS containing 0.1% Tween 20.

Fig. 3. Subcellular localisation of endogenous thiolase 18 h after fusion of fibroblast cell lines. (A) Zellweger cells X CDP1 cells; (B) CDP1 X CDP2; (C) CDP1 X MCHE 85AD; (D) CDP2 X MCHE 85AD; (E) CDP3 X MCHE 85AD; (F) CDP4 X MCHE 85AD.
Antigen-antibody complexes were visualised using the ECL kit from Amersham.

3. Results

Fibroblasts from each of the patients were tested for their ability to import reporter proteins for the type 1 and 2 peroxisome targeting signals (luciferase and prethiolase-CAT, respectively) into their peroxisomes. A particulate pattern of labelling was seen in control fibroblasts after expression of both luciferase (Fig. 1A) and prethiolase-CAT (Fig. 1C); both these reporter proteins have previously been shown to co-localise with endogenous peroxisomal proteins [8]. The PTS1 reporter protein luciferase has previously been shown to be imported into peroxisomes in RCDP cells [8]. This reporter protein is also targeted to peroxisomes in cell lines CDP1-4 [13]. Fig. 1B shows the expression pattern in CDP1 cells.

In contrast to the PTS1 reporter protein, expression of the PTS2 reporter protein in CDP1 cells gave rise to a diffuse pattern of labelling resembling a cytoplasmic localisation (Fig. 1D). Labelling patterns similar to the latter were observed with cell lines CDP2-4 (not shown) and have also been observed in cells derived from classical RCDP patients [8]. The labelling patterns seen after expression of prethiolase-CAT were similar to those seen when endogenous thiolase was visualised using a monoclonal antibody (Fig. 1E,F).

Thiolase import was assessed further by determining the accessibility of endogenous peroxisomal thiolase to protease digestion. Precursor thiolase is protected from protease digestion in fibroblasts from a neonatal adrenoleukodystrophy patient (Fig. 2, upper panel) in which import of precursor thiolase has been shown to be intact [8]. In all of the CDP patients (of which CDP1 is shown in Fig. 2, lower panel), precursor thiolase is degraded by proteinase K, presumably because of its failure to be imported into peroxisomes.

The genetic relationship between the four CDP patients and a classical RCDP patient was investigated by complementation analysis after somatic cell fusion of different combinations of fibroblasts. As has been shown by Heikoop et al. [13], the appearance of punctate fluorescence using a monoclonal anti-thiolase antibody can be taken as a morphological criterion to indicate complementation. When fibroblasts from a Zellweger patient were fused with CDP1 fibroblasts, a punctate pattern of fluorescence was seen in the heterokaryons (Fig. 3A). Similar results were obtained after fusion of CDP2-4 with ZS cells (not shown). In contrast to the fusions with ZS fibroblasts, no punctate fluorescence could be observed when different, pairwise combinations of fibroblasts from the CDP patients were fused with each other, indicating that cell lines CDP1-4 all belong to the same complementation group. Fig. 3B shows the labelling pattern after fusion of cell lines CDP1 and CDP2.

In order to investigate whether the CDP patients are deficient in the same gene as that deficient in classical RCDP patients, cell lines CDP1-4 were each fused with a representative cell line from the large complementation group that contains fibroblasts from 9 classical RCDP patients [13]. No punctate fluorescence was seen after fusions between CDP and classical RCDP cell lines, indicating that all the cell lines are deficient in the same gene. Fig. 3C-F shows the pattern of labelling after pairwise fusion of cell lines CDP1-4, respectively, with a classical RCDP cell line (MCHE 85AD).

4. Discussion

The non-rhizomelic chondrodysplasia punctata patients analysed in this study have previously been shown to possess the biochemical abnormalities characteristic of RCDP patients [9-12]. We have compared fibroblasts from non-rhizomelic CDP patients to those from a classical RCDP patient with respect to their ability to import the PTS1-containing protein luciferase and the PTS2-containing protein thiolase. We have also investigated the genetic relationship between non-rhizomelic CDP patients and a classical RCDP patient using complementation analysis after somatic cell fusion. Fibroblasts from the classical RCDP patient have already been shown to belong to the same complementation group as 8 other patients with the clinical manifestations of RCDP [13].

The ability of CDP and RCDP cells to import luciferase shows that the PTS1 import route is intact in these cells. Analysis of (i) the subcellular localisation of a reporter protein for the thiolase import route using immunofluorescence and (ii) the accessibility of endogenous thiolase in permeabilised cells to protease degradation has shown that precursor thiolase in both RCDP and non-rhizomelic CDP fibroblasts failed to be imported into peroxisomes. We conclude from the above that the non-rhizomelic CDP patients cannot be distinguished from the classical RCDP patient either with respect to their ability to import the PTS1-containing reporter protein luciferase, or with respect to their inability to import PTS2-containing peroxisomal thiolase.

It was therefore of interest to investigate the genetic relationship between the RCDP and the non-rhizomelic CDP patients. The failure of punctate thiolase labelling to appear after fusion of various combinations of non-rhizomelic CDP fibroblasts and RCDP fibroblasts (in contrast to when Zellweger fibroblasts were used as a fusion partner) indicates that the non-rhizomelic CDP and RCDP fibroblasts all belong to the same complementation group. It is striking that defects within one and the same gene can give rise to both the rhizomelic and the non-rhizomelic forms of CDP. It is possible that the mutations present in the non-rhizomelic CDP patients give rise to a protein product with higher residual activity than that found in the
more severe RCDP patients, as at least one of the CDP patients [9] had higher DHAP-AT activity than that found in RCDP patients. Support for this possibility is provided by Wanders et al., who have described two patients [17,18] who showed all the clinical features of RCDP but lacked the tetrad of biochemical abnormalities usually found in RCDP patients: in both patients, phytanic acid oxidation was normal, and thiolase was present in its mature form inside peroxisomes. However, both patients showed a deficiency in plasmalogen biosynthesis: in one patient, the first enzyme required for plasmalogen biosynthesis was deficient (DHAP-AT) [17], while the other patient lacked only the second enzyme [18]. These results strongly suggest that the pathogenesis of RCDP is directly related to the inability to synthesise plasmalogens [17,18].

The finding that patients suffering from non-rhizomelic and rhizomelic CDP have a deficiency in the same gene indicates that the former is simply a milder form of the latter. A complementation group which includes patients suffering from the peroxisomal disorders Zellweger Syndrome, Infantile Refsum’s disease and neonatal adrenoleukodystrophy shows that phenotypic heterogeneity within a single complementation group is not unprecedented [15,19–21].

References