Peroxisomes and Peroxisomal Disorders

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Editorial

Peroxisomal Disorders — A Pathobiochemical Lesson on Peroxisomal Function

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During their classic studies on the separation of lysosomes by centrifugation of rat liver homogenates, De Duve and his group (1) observed that certain oxidative enzymes, such as D-amino acid oxidase and uric acid oxidase which produce hydrogen peroxide, and catalase which attacks hydrogen peroxide, were always found in a discrete fraction which could be separated from lysosomes and mitochondria. These enzymes were believed to be associated with special organelles called peroxisomes and shown to be identical to the organelles first described by Rhodin (2) as microbodies in 1954.

Peroxisomes are mainly spherical organelles bounded by a trilaminar unit membrane and containing a fine granulär matrix. It is believed that peroxisomal proteins are formed on the endoplasmic reticulum and that peroxisomes are then produced by a budding process. Peroxisomes are never formed de novo and a cell must contain at least one single peroxisome. It is now well established that peroxisomes exhibit important functions in mammalian lipid and amino acid metabolism. These functions include steps in the biosynthesis of plasmalogens and bile acids and fatty acid β-oxidation, particularly those of very long chain fatty acids.

The interest in peroxisomes has been greatly stimulated by two lines of investigations, i.e. the discovery of the remarkable proliferative effect of several hypolipidaemic drugs such as clofibrate on hepatic peroxisomes, and the recognition of several genetic human disorders in which peroxisomes are either totally absent or abnormal.

In 1965 Zellweger (3) in Iowa City and Bowen (4) at Johns Hopkins described certain neonates, which develop characteristic symptoms such as muscular hypotonia, seizures with neonatal onset, psychomotor retardation, craniofacial dysmorphism and ocular involvement. Similar disorders in 5 siblings were described by Passarge & McAdams (5), demonstrating disorders in brain, liver and kidney, who coined the term cerebro-hepato-renal syndrome. In 1969 Opitz (6) proposed the eponym Zellweger syndrome for the case first described at Iowa City in honour of his former teacher. In 1973 Goldfischer and coworkers (7) demonstrated that patients with Zellweger syndrome show a reduced number or absence of peroxisomes.

Studies on the peroxisomal fatty acid oxidase system helped to clarify the pathogenesis of Zellweger syndrome. The enzymes involved in the peroxisomal β-oxidation system were purified and characterized by Hashimoto (8). In principle both the starting compound and the product of β-oxidation are identical in peroxisomes and mitochondria, but the enzymes involved are totally different. The peroxisomal pathway, which is most important for the oxidation of very long chain fatty acids, does not involve carnitine. The activated fatty acid is first oxidized by acyl-CoA oxidase, with the production of hydrogen peroxide. The unsaturated activated fatty acid is hydrated to its 3-hydroxy derivative and oxidized to 3-oxo-acyl-CoA by a bifunctional enoyl-CoA hydratase/3-hydroxy acyl-CoA dehydrogenase. The 3-oxo-acyl CoA is finally split by 3-oxo-acyl CoA thiolase. Dutch authors were able to show by immunoblotting that Zellweger and neonatal adrenoleukodystrophy postmortem liver and cultured fibroblasts lack the bifunctional enzyme. Acyl-CoA oxidase is also deficient in Zellweger syndrome and the 3-oxo-acyl CoA thiolase fails to be processed normally. The deficiency of these enzymes accounts for the accumulation of very long chain fatty acids characteristic of Zellweger's syndrome.

Several other peroxisomopathies have been found, which are of current interest because they occur more commonly than had hitherto been recognized. Their study provides an opportunity to learn more about the role of peroxisomes. Peroxisomal disorders recognized during recent years have been grouped into three general categories (9—11).

In the first group, peroxisomes are totally lacking or at least strongly reduced in number. This group includes the cerebro-hepato-renal Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum's disease and hyperpipecolic acidemia. Patients lack the capacity to synthesize plasmalogens and to oxidize very long chain fatty acids or phytanic acid in Refsum's disease, and show abnormally high levels of bile acid intermediates.
and of pipecolic acid. Patients rarely survive early childhood and show severe neurological defects and multiple malformations.

In the second group peroxisomal structure is intact, but several peroxisomal enzymes are functionally deficient. This group includes the pseudo-Zellweger syndrome, characterized by an isolated 2-oxo-acyl CoA thiolase deficiency, and the rhizomelic form of chondrodysplasia punctata, characterized by deficient plasmalogen synthesis.

Finally, in a third group of peroxisomopathies peroxisomal structure is intact and the defect is thought to be due to a mutation affecting a single peroxisomal enzyme. This group includes X-linked adrenoleukodystrophy, characterized by a lack of peroxisomal very long chain fatty acid oxidation, adult Refsum's disease with its typical lack of phytanic acid oxidation, and acatalasemia.

Clinical chemical assays suitable for the diagnosis of peroxisomal disorders include the determination of increased plasma levels of very long chain fatty acids, gas chromatography of dimethylacetals in plasma, measurement of acyl-CoA : dihydroxycetonephosphate acyltransferase activity and of the concentration of bile acid intermediates in plasma. The demonstration of elevated pipecolic acid concentrations in serum and urine are also of diagnostic value, but this abnormality is not invariably present and other conditions may also cause elevated pipecolic acid concentrations. The gas chromatographic assay of plasma phytanic acid concentrations is useful for the diagnosis of Refsum's disease.

With the exception of Refsum's disease, which can be successfully treated by dietary restriction of phytanic acid, therapy of peroxisomopathies is still in the experimental stage. It would be a reasonable assumption to treat patients showing a reduced number of peroxisomes with substances inducing their proliferation. However these trials were unsuccessful in Zellweger patients. This can easily be explained by the fact that peroxisomes never form de novo. In addition, experiments with peroxisomal proliferators such as clofibrate have never been successfully performed in primates. Recently a new hypolipidaemic drug has been tested which is also active in Rhesus monkeys; it has not yet been applied in patients with peroxisomopathies. In adrenoleukodystrophy, dietary restriction of very long chain fatty acids alone fails to alter very long chain fatty acids in plasma. This is not surprising since very long chain fatty acids are not only derived from dietary sources but also from endogenous biosynthesis. However, a partial normalization of very long chain fatty acid concentrations in plasma and red blood cells can be achieved by reducing both dietary very long chain fatty acids and their endogenous synthesis. This has been achieved by the administration of trioleate glycerol which competitively reduces the elongation of saturated fatty acids with 16 and 18 carbon atoms. Finally, a reduction of very long chain fatty acids in plasma can be obtained by the transplantation of enzymatically competent bone marrow cells.

At present the question of the phenotypic and genotypic heterogeneity of peroxisomopathies is the focus of research. Roscher and his group in Munich have approached this problem by complementation analysis using a somatic cell hybridization technique. This is performed by bringing together the defective gene of each mutant in the same cell by cell fusion, and testing for the restoration of function. The results obtained so far are consistent with the hypothesis that at least 4 different gene mutations may cause phenotypes clinically described as neonatal adrenoleukodystrophy and/or Zellweger's syndrome (12).

A number of questions remain unanswered in peroxisomal research. They will be answered by a close cooperation between clinicians and clinical biochemists.

References


Morphology and Metabolic Functions of Peroxisomes

Zellweger, H.

The Origin of Peroxisomes and Peroxisomal Disorders

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In September of this year (1988) it will be exactly 60 years since I entered medical school. What did the anatomist tell us about the cell? In brief, nucleoplasm, protoplasm, cell membrane, Golgi apparatus and an occasional inclusion body. All other organelles that I am going to enumerate were unknown, because the technique for recognizing them, namely the electron microscope, had not been discovered in 1928. It was many years before that technical refinement was achieved which made it possible to distinguish intracellular organelles such as mitochondria, lysosomes and later peroxisomes. The explosive development of biochemistry, including the ability to isolate these organelles in the late 1950s and subsequent decades, led to the identification of their various functions. These developments are mainly to the credit of Emil Palade and Christian Rene de Duve, who both earned the 1975 Nobel prize for physiology and medicine for their accomplishments. A new nosological taxonomy arose from those studies; workers began to distinguish between mitochondrial cytopathies, lysosomal disorders and since 1973 also peroxisomal disorders.

Turning now to the peroxisomal disorders, their history can be divided in 4 phases.


Phase 2: Recognition of the absence of peroxisomes in liver and renal tubular cells in Zellweger syndrome and the discovery of diversified metabolic abnormalities in Zellweger syndrome due to the absence of peroxisomes (1a).

Phase 3: Recognition of other peroxisomal disorders other than Zellweger syndrome beginning in 1978.

Phase 4: Etiological distinction of various peroxisomal disorders, by cell fusion and heterokaryon analysis.

The history of the peroxisomal disorders has been described in recent reviews by Monnens & Heymanns (1b), Moser (2), Schutgens et al. (3), Zellweger (4). I refer to those reviews and shall concentrate on phase 1 in which I was intimately involved.

In the early 1960s the medical profession knew very little about genetics, or at least did not apply genetics to their clinical practice. Thus referrals to the genetic clinic and to the chromosomal laboratory, which in the University of Iowa had been opened in spring 1960, were very scanty. One of my friends rightly stated: «If you want to popularize the genetic clinic you have to teach genetics first to the medical faculty». With the help of funds from the medical dean, I was able to organize a postgraduate course in genetics and to invite some of the outstanding geneticists of that time to lecture to our faculty. One of them was Victor McKusick from Johns Hopkins University. Preceding his lecture I presented to him the puzzling case of a 3 month infant, who had all the signs and symptoms of the Zellweger syndrome, which will be described in the presentations of Drs. Sylvia Sockler and J. M. Powers. Neither McKusick nor I could classify the case, yet he remembered two cases at Hopkins, who presented very similar course and
symptomatology. He suggested that we publish these cases together, which led to the publication by Bowen et al. (5). The term cerebro-hepato-renal syndrome was coined by Passarge & McAdams in 1967 (6). These authors had seen the syndrome in 5 siblings, but only the fifth case made the authors aware that they were dealing with a particular syndrome. And this is what happened quite frequently in the 1960s and early 1970s. I know of many cases through correspondence with various medical centres throughout the world, which have never been diagnosed as Zellweger syndrome. Our 1964 case was the third sibling of that particular family, who presented with the typical picture. The first two siblings with similar symptomatology had died in the first two weeks of their lives, i.e., too early to make a diagnosis. This has implications with respect to the incidence of the condition. Danks et al. (7) estimated that one in 100 000 newborns would be affected with Zellweger syndrome. Our experience in Iowa and also the experience of the Dutch authors suggest a higher frequency, perhaps between one in 25 000—50 000 newborns. How did the term Zellweger syndrome arise, since Bowen signed as the senior author in the 1964 paper l.c. (5)?

In 1969 Opitz (8) analysed the hitherto reported cases and came to the conclusion that the Baltimore cases were different from the Iowa case. Thus, in deference to his former teacher, he proposed the eponym Zellweger syndrome for the Iowa case. This eponym has now been fairly generally accepted. Professional honesty, however, compels me to say that I was indeed not the first to describe the syndrome. Cornelia de Lange (9) described in 1948 a case which showed all the criteria of rat hepatocytes. Since then rat hepatic microbodies have been studied most extensively. In 1965 de Duve (3) introduced the term peroxisome and his concept of peroxisomes, the association of H2O2-producing oxidases and catalase within a membrane-bound organelle. In the same year it was shown that mainly microbodies are present in purified peroxisomal preparations; the term peroxisome has been used since that time.

Peroxisomes are mainly spherical organelles with diameters up to light microscopic dimensions (0.5 µm to 1.5 µm in hepatocytes, up to 0.3 µm in proximal kidney tubules). The organelles are bounded by a trilaminar unit membrane (6—7 nm thick) and they contain homogeneous or faintly granular matrix material of moderate electron density. Most mammalian peroxisomes contain a centrical or eccentric density which may display crystalline patterns. These structures are called cores or nucleoids, their presence coincides with the occurrence of urate-oxidase in the respective tissue. Membranous continuities (connecting channels) may be seen in favourably oriented sections between peroxisomes and smooth endo-
The quality of the science presented at the Seventh Workshop on Vitamin D was outstanding. Major developments were made in understanding of Vitamin D on many research frontiers, including those of the chemists, biochemists, physiologists as well as clinicians who attended the meeting and who are actively conducting research in various aspects of the Vitamin D endocrine system.

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plasmic reticulum and between peroxisomes themselves. From these observations it was concluded that peroxisomes do not exist as individual entities but represent reversible dilatations of smooth endoplasmic reticulum.

Identification of peroxisomes in the light and electron microscope is significantly facilitated by means of diaminobenzidine histochemistry: diaminobenzidine is oxidized to an insoluble osmiophilic polymer via the peroxidatic activity of catalase. Inhibition experiments with aminotriazole can be used to differentiate between the peroxidatic activity of catalase and other haem proteins.

In addition to classical peroxisomes with nucleoids, smaller organelles devoid of cores were also found in rat hepatocytes. These particles are significantly smaller (0.1 — 0.3 mm in diameter) and of irregular shape: spherical as well as polygonal or elongated cylinders. Diaminobenzidine histochemistry showed that these particles contain catalase. These smaller catalase-positive particles were named microperoxisomes. Extensive morphological investigations confirmed that microperoxisomes are ubiquitous cell organelles. They are particularly frequent in the glandular epithelium of lipid secreting glands (Harder's gland, uropygeal gland, preputial gland, sebaceous gland).

Serial sectioning substantiated the assumption that peroxisomes are interconnected with one another by delicate channels (classical peroxisomes as well as microperoxisomes). In addition, peroxisomes are often seen to form clusters. Electron microscopy of thick (1.5 mm) sections from diaminobenzidine stained material illustrates the intricate tridimensional network of interconnected microperoxisomes. These morphological observations substantiate the hypothesis of a »peroxisomal reticulum« presented by Lazarow (4). The concept was formulated to interpret chemical results on the distribution of newly synthesized peroxisomal enzymes. Peroxisomes are assumed to be interconnected, either really in space or in time by alternating fusion and fission processes. Therefore the model is a dynamic one and not all peroxisomes but is also able to provide acetyl-CoA as a fuel for mitochondrial thermogenesis. Examples of peroxisomal oxidases producing hydrogen peroxide are the β-oxidation enzyme, fatty acyl-CoA oxidase, and urate oxidase, the latter being found only in non-primate mammals. Xanthine oxidase was detected last year in

The reliable and convincing demonstration of this new subcellular compartment awaits improvement in techniques for the elucidation of its fine structure.

References


Kramar, R., K. Kremsen and H. Schön

Metabolic Function of Peroxisomes

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Peroxisomes form hydrogen peroxide by the action of certain oxidases, mostly flavoproteins, and dismutate it into oxygen and water by the action of catalase. This classical scheme of De Duve also includes other hydrogen donors which can be oxidized by the peroxidatic reaction of catalase. When present in appropriate concentrations, substrates like lower alcohols, for example methanol, are dehydrogenated.

Thus, the very simple respiratory system of the peroxisomes leads to the oxidation of substrate-bound hydrogen to water. In contrast to the mitochondrial respiratory chain, no ATP is produced in the peroxisomal pathway, and the free energy of electron transport is dissipated as heat. In addition, peroxisomal NADH, serving as a reductant for oxidized substrates, particularly for glyoxalate, may also be oxidized with loss of the available energy as heat. Alternatively NADH easily leaves the peroxisomes and reduces cytochrome b on the cytosolic side of the membrane, as demonstrated in our laboratory some years ago.

By these energy-wasting reactions peroxisomes might play a role in the control of body heat by chemical thermogenesis. In the brown fat of the cold-adapted rat we found an increased peroxisomal fatty acid β-oxidation, which is not only energy dissipating per se, but is also able to provide acetyl-CoA as a fuel for mitochondrial thermogenesis. Examples of peroxiso- mal oxidases producing hydrogen peroxide are the β-oxidation enzyme, fatty acyl-CoA oxidase, and urate oxidase, the latter being found only in non-primate mammals. Xanthine oxidase was detected last year in
the cores of rat liver peroxisomes. Thus, the central part of purine degradation in mammals might be allocated to peroxisomes. The question remains as to where xanthine oxidase is located in human peroxisomes, which do not possess a crystalline core. In carp liver which is also devoid of peroxisomal cores, the enzyme seems to be soluble.

The presence of xanthine oxidase poses the problem of detoxication of the superoxide radical which is formed by this enzyme, but not by the other peroxisomal oxidases. Thus, peroxisome proliferators like hypolipidaemic drugs may be responsible for increased production of dangerous radicals. In addition, the elevated hydrogen peroxide formation due to the induction of fatty acyl-CoA oxidase promotes the generation of extremely toxic hydroxyl radicals:

\[ \text{H}_2\text{O}_2 + \text{O}^- \rightarrow \cdot\text{OH} + \text{OH}^- + \text{O}_2 \]

The physiological function of D-amino acid oxidase and L-α-hydroxy acid oxidase remains somewhat obscure. In mammals D-amino acids are encountered only in negligible quantities, originating from the intestinal flora. Glycine, which of course is turned over by D-amino acid oxidase, reacts only sluggishly, so that the transformation of glycine into glyoxylate by D-amino acid oxidase does not lie on the main route of glycine degradation. On the contrary, in peroxisomes glycine is formed from glyoxylate by transamination with alanine and other amino acids by the peroxisomal transaminase described by Tolbert & Noguchi. This reaction is important in the detoxication of glyoxylate, preventing hyperoxaluria. With regard to the oxidation of L-amino acids, the rat kidney L-α-hydroxy acid oxidase B is able to deaminate some L-amino acids. No extensive search for peroxisomal L-amino acid oxidases in different mammalian species has yet been performed.

L-Pipecolic acid, a rare cyclic amino acid, exhibiting γ-aminobutyric acid (GABA)-like neurotransmitter functions, is elevated in blood and excreted in relatively high amounts in the urine of patients suffering from those peroxisomal disorders characterized by virtual absence of peroxisomes, namely cerebro-hepato-renal Zellweger syndrome, infantile Refsum's disease and neonatal adrenoleukodystrophy (for review see I.e. (1)). Presumably pipecolic acid is dehydrogenated in the peroxisomes by a hydrogen peroxide producing oxidase, which might be impaired in the mentioned disorders (2).

In conclusion a short listing of the peroxisomal functions connected with lipid metabolism is given (3):

1. In addition to the degradation of long chain fatty acids, the shortening of prostaglandin F₂α and of the cholesterol side chain during bile acid formation are β-oxidative sequences connected with peroxisomes. This is also true for the degradation of dicarboxylic acids.

2. The α-oxidation of phytanic acid and possibly its further β-oxidative degradation are due to peroxisomes. 3-Hydroxy-3-methyl-glutaryl-CoA reductase, the key enzyme, and presumably other enzymes of cholesterol biosynthesis, exist in a peroxisomal moiety. NADPH consumed in the mentioned reaction and in the dihydroxyacetone phosphate pathway of glycerolipid biosynthesis might be provided by glucose-6-phosphate dehydrogenase which has been detected in peroxisomes.

3. Recently the formation of another isoprenoid, namely dolichol, was ascribed to peroxisomes.

4. Peroxisomes are indispensible in forming saturated ether lipids and plasmalogens, since dihydroxyacetone phosphate acyltransferase, 1-alkyldihydroxyacetone phosphate synthase and alkylidihydroxyacetone phosphate reductase are located in the peroxisomal membrane.

Acknowledgement
We thank the Anton Dreher Gedächtnisschenkung for financial support.

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patients with Zellweger syndrome lack almost totally the ability to perform this reaction, but are able to convert normally trihydroxycholestanate to trihydroxycholestanolic acid, in the mitochondria (2).

It is known that in Zellweger disease, the absence of morphologically demonstrable peroxisomes, though not necessarily meaning total absence of peroxisomal enzymes, is accompanied by decreased peroxisomal reactions and serves as an explanation for the observed symptoms. Moreover it was shown that the residual small amount of β-oxidation type side chain cleavage activity in hepatocytes of these patients was insensitive to KCN, a characteristic of peroxisomal β-oxidation.

Hep G2 cells are a highly differentiated human hepatoma cell line, and numerous reports describe their metabolism. Everson & Polokoff (3) recently showed that Hep G2 cells are able to synthesize bile acids, but in an abnormal pattern. Besides a defect in conjugation, these cells are specifically deficient in the side chain cleavage that transforms trihydroxycholestanate into cholic acid. It would be a strong argument supporting the hypothesis of peroxisomal localization of the side chain cleavage reactions during bile acid synthesis, if the peroxisomes of Hep G2 cells were found to be defective.

To this end, we have shown by immunofluorescence techniques that peroxisomes are morphologically demonstrable in Hep G2 cells in a similar pattern to that of isolated hepatocytes. Their enzymes sediment in the same way as hepatocyte peroxisomal enzymes, but there is a striking difference in the specific activity of catalase when compared with isolated hepatocytes. Other peroxisomal enzyme activities, e.g. palmitoyl-CoA oxidase seem to be normal.

Analysis of the catalase protein by Western blot reveals a normal form of $M_r = 60 \times 10^3$ and a lower molecular weight form of approximately $40 \times 10^3$. Electrophoretic separation under non-reducing and non-denaturing conditions separates catalase again into two moieties, including an enzymatically inactive, lower molecular weight form.

These findings support the hypothesis that peroxisomes are active in bile acid formation, since defective formation of bile acids is paralleled by a defective peroxisomal system found for the first time in Zellweger cells and now in Hep G2 cells.

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Paltauf, F. and A. Hermetter

Peroxisomes in the Biosynthesis of Plasmalogens

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Several years ago Hajra and coworkers (1) reported that peroxisomes, which were thought to harbour mainly oxidative enzymes of substrate conversion, contain enzymes involved in the biosynthesis of alkylglycerophospholipids.

Two types of ether glycerolipids have been identified as constituents of mammalian cell membranes: derivatives of 1-O-alkyl-sn-glycerol and of 1-O-1’-(Z)-alkenyl-sn-glycerol. The latter are termed plasmalogens and constitute, mainly in the form of ethanolamine and choline glycerophospholipids, major components of several types of cell membranes. Especially rich in plasmalogens are the central nervous system, muscle, heart, kidney, blood cells, bone marrow, intestinal epithelial cells, etc (for a review, see I.e. (2)). Interestingly, plasmalogens are rarely found in plant cells, fungi or aerobic eubacteria. The subcellular distribution of plasmalogens varies between different types of cells; ether lipids are not enriched in peroxisomes.

The overall reaction sequence leading to the formation of plasmalogens is depicted in the scheme. Only two reactions are unique to ether lipid biosynthesis, namely the formation of the ether bond by alkylidihydroxyacetone phosphate synthase and the alkylglycerophosphoethanolamine desaturase, which forms the vinyl ether linkage of plasmalogens. All other enzymes are the same as those involved in the biosynthesis of diacylglycerophospholipids. Two of the enzymes involved, dihydroxyacetone phosphate acyltransferase and alkyl dihydroxyacetone phosphate synthase, are located in peroxisomes; more precisely, they are membrane-bound, facing the luminal side of peroxisomes. Alkyl dihydroxyacetone phosphate reductase is also associated with peroxisomes, but faces the cytosol.

The assay of the two peroxisomal enzymes of ether lipid biosynthesis is of value to the biochemical diagnosis of a group of inherited disorders, which are
Constituting the key role of peroxisomes in ether lipid biosynthesis, the question arises of whether the cells cultured with a high capacity to synthesize other lipid types can be applied to the biochemical and biophysical properties. Several such differences have been identified, which preferentially hydrolyze the acyl ester group in position as the Ca^2+ -independent enzymes from rabbit lung microsomes.

Experiments are in progress to study in more detail the effects of plasmalogen depletion on membrane function, e.g., transport processes or receptor activity. Membrane fluidity is enhanced by plasmalogen deficiency, which increases our understanding of the role of ether lipids in membrane function. Furthermore, a preferential uptake of exogenous plasmalogens by plasmalogen-depleted fibroblasts was observed (own results, unpublished). Pretreatment with phorbol ester abolishes these effects.

1) Some ether lipids are biologically active, the most prominent example being the so-called platelet activating factor. 2) Ether and diacylglycerophospholipids differ in physical properties. For example, the ether bond resists chemical and enzymatic hydrolysis. On the other hand, phospholipases A2 of dog liver mediate the hydrolysis of ether lipids. As have been identified, which preferentially hydrolyze the acyl ester group in position as the Ca^2+ -independent enzymes from rabbit lung microsomes.

3) Ether lipids and their diacyl counterparts differ with respect to chemical and biochemical properties. For example, the ether bond resists chemical and enzymatic hydrolysis. On the other hand, phospholipases A2 of dog liver mediate the hydrolysis of ether lipids. As have been identified, which preferentially hydrolyze the acyl ester group in position as the Ca^2+ -independent enzymes from rabbit lung microsomes.

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In other words, what is the biological significance of ether lipids? Three aspects should be considered in this context:

1. The role of peroxisomes as a primary locus of ether lipid synthesis of vital importance to cellular function?
2. The role of peroxisomes as a primary locus of ether lipid synthesis of vital importance to cellular function?
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References


Many compounds have been found to induce peroxisome proliferation in the liver of rats and some other animal species. These agents include hypolipidaemic drugs such as clofibrate, nafenopin, fenofibrate, gemfibrozil etc., drugs from various other pharmacological classes, e.g. acetasalicylic acid, an uricosuric drug, certain steroids, phenoxyacetic acid herbicides and plasticizers such as diethylhexylphthalate, or diethylhexyladipate. Obviously the compounds are structurally quite diverse. A common structural property of many compounds or of their active metabolites appears to be a carboxyl group a few atoms apart from a somewhat bulky, lipophilic residue. Although most research activity has been devoted to induction of peroxisomes and some peroxisomal enzymes, other changes occur in the liver, and these may be important in understanding the effects and mechanisms of peroxisome proliferators. These changes include increases in mitochondrial acyl carnitine transferase, in microsomal cytochrome P-452, in aldehyde dehydrogenase, in malic enzyme and glucose-6-P-dehydrogenase. Finally, there is a pronounced stimulation of liver growth and mitotic activity in the liver. Thus it appears that a complex gene programme, rather than increases of a few specific enzymes, is expressed by peroxisome proliferators.

In long-term animal experiments many of the (more potent) peroxisome proliferators were found to produce carcinomas, mainly in the liver. This was surprising because the agents do not exhibit detectable mutagenic/genotoxic activity as do carcinogens of the nitrosamine or aromatic hydrocarbon type. Peroxisome proliferators are therefore classified as “non-genotoxic carcinogens”, a class of agents also comprising microsomal enzyme inducers such as phenobarbital or DDT, certain steroid hormones, etc.

For the assessment of health risks to humans exposed to peroxisome proliferators it is most important to understand the mechanism(s) by which these agents produce liver tumours in animals. Cancer in many cases is known to occur through a sequence of stages called initiation, promotion and progression.

Chemical agents can cause tumour formation by interference with any of these stages. Thus, studies with phenobarbital and some steroid hormones have shown that these compounds most likely accelerate (promote) the development of prestage of cancer into manifest tumours. Such prestage were shown to occur spontaneously. Evidence suggesting a promotion effect of phenobarbital is based on induction of growth and phenotypic expression in putative preneoplastic cell foci in rat liver. So far it is not clear whether peroxisome proliferators lead to tumours through a

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Stimulation of Peroxosome Proliferation by Drugs and Carcinogenesis

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similar mechanism of action. Biochemical and morphological liver changes in response to these agents, which may be relevant to carcinogenesis, will be presented in the following two papers.

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Morphological Aspects of Hepatocarcinogenesis by Hypolipidaemic Agents in Rats

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Peroxisome-inducing agents (1, 2) produce hepatocellular carcinoma in long-term animal bioassays. These chemicals have not been found to be DNA-reactive and thus may be so-called “tumour promoters” enhancing tumour development only in hepatocytes that have been “initiated” spontaneously or by genotoxic carcinogens. Foci of phenotypically altered liver cells are regarded to be both progenies of initiated cells and stages of hepatocellular cancer.

Putative preneoplastic foci were induced in the livers of 21 female Wistar rats by a single oral application of the potent hepatocarcinogen aflatoxin Bj (5 mg/kg body weight). After a recovery period of 3 weeks, 9 animals were fed a diet containing the peroxisome inducer nafenopin with an adjusted daily dose of 100 mg/kg body weight. The remaining 12 animals were fed a normal chow and served as control.

After 70 weeks of treatment, 9 liver tumours had developed in 4 out of 9 nafenopin-treated rats, whereas in 12 control livers only 1 tumour could be found. Thus, when administered after a genotoxic carcinogen, nafenopin showed tumour enhancing activity. This supports the hypothesis of tumour promotion by peroxisome proliferators.

Phenobarbital causes an increased expression of phenobarbital-inducible enzymes in liver foci. Are there analogies for peroxisome proliferators, e.g. does nafenopin cause an increase of nafenopin-inducible enzymes in focal tissue? How do focal hepatocytes cope with the functional load induced by proliferation of peroxisomes? Do foci adapt by increasing their expression of peroxisomal enzymes or are they resistant by decreasing their response to peroxisome inducers?

Enzyme expression was studied by immunohistochemistry. The selected enzymes were:

- peroxisomal acyl-CoA-oxidase, bifunctional protein, and thiolase, which degrade fatty acids by β-oxidation
- microsomal cytochrome P-452, a cytochrome P-450 isoenzyme induced by peroxisome proliferators, which contributes to lipid metabolism by catalyzing ω-hydroxylation of fatty acids.

The immunohistochemical localization of the selected enzymes was carried out on liver sections.

Non-focal parts of livers of nafenopin-treated rats showed an increased immunoreaction for all the enzymes examined, especially in the pericentral part of the liver. In control livers the staining was homogeneously distributed within the lobule. Irrespective of treatment with nafenopin, however, almost all foci revealed a decreased immunoreaction for all enzymes in comparison with surrounding hepatocytes. No relation between focal size and degree of enzyme reduction could be found, neither in controls nor after nafenopin treatment. Thus, it seems unlikely, that foci lose enzymes induced by peroxisomal proliferation during their growth.

The question arises of whether a decrease in peroxisomal and microsomal fatty acid metabolism is the specific and necessary effect for hepatocarcinogenesis by peroxisome inducing agents. Only the tumour data

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favour the assumption that peroxisome proliferators, like nafenopin, are tumour promotores. Their effects on tumour prestages, however, do not seem to support this assumption. Further studies are therefore needed to elucidate the mechanism of hepatocarcinogenesis by peroxisome proliferators.

References


Huber, W.

Biochemical Effects of Peroxisome Proliferators with Respect to Carcinogenesis

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Carcinogenesis after peroxisome proliferator treatment seems to differ from traditional models. Therefore it has often been described as a “novel form of carcinogenesis”. The mechanism by which this treatment produces cancer is unclear. Two hypotheses most frequently found in literature are discussed:

1. Hydrogen peroxide production

Peroxisome proliferators were not found to be genotoxic in the commonly applied test systems. Nevertheless, DNA could be damaged “indirectly” by intermediates formed during peroxisome proliferator-enhanced metabolic processes. Most often mentioned among those intermediates are hydrogen peroxide and oxygen radicals. Under physiological conditions hydrogen peroxide is formed in the peroxisome as a by-product of β-oxidation and degraded immediately by catalase. Peroxisome proliferators increase the activity of hydrogen peroxide-forming peroxisomal β-oxidation 6 to 20-fold, while they only double the detoxifying catalase activity. The resulting imbalance increases the intracellular concentration of hydrogen peroxide.

Hydrogen peroxide readily peroxidizes macromolecules. On the one hand, the macromolecules activated in this way could react with DNA, thus leading to cancer. A direct reaction of DNA with hydrogen peroxide, however, seems unlikely. Secondarily, membrane lipids are an excellent target for peroxidation. This can lead to membrane decay, subsequent cell death and a growth stimulus for the more resistant cells. If this process gets out of control it could also cause cancer.

The same effects can be caused by oxygen radicals, which, after peroxisome proliferation, show enhanced formation through oxidative reactions (cytochrome P-450) or from hydrogen peroxide.

Enzymes that protect against hydrogen peroxide and oxygen radicals, or repair the damage, are only moderately increased (catalase) or even reduced (superoxide dismutase, glutathione-dependent peroxidase) by peroxisome proliferators. The pathway from hydrogen peroxide to cancer would thus clearly overwhelm these defense mechanisms.

Results reported in the literature do not permit a clear decision as to whether or not this hypothesis is tenable. Some support is found:

1. The pigment lipofuscin, which is reported to be a conglomerate of peroxidized lipid membrane degradation products, is increased. This suggests that after peroxisomal proliferation lipid peroxidation can indeed reach a detectable damage level.
2. Peroxisome proliferator-treated rats were more sensitive to additional peroxidative treatment.
3. Decrease of protective enzymes.
4. Increase in microsomal cytochrome P-450.
5. An increase in permeability of the peroxisomal membrane for hydrogen peroxide was observed, making it easier for hydrogen peroxide to leave the peroxisome and to reach its target molecules.

In spite of these supporting facts, contradictory results were obtained:

1. Hydrogen peroxide was positive in several mutagenicity tests, but the test was performed under conditions very different from those in vivo. Peroxisome proliferation was therefore successfully performed in culture, leading to hydrogen peroxide production. No change of DNA-repair however was observed in comparison with controls.
2. The herbicide paraquat is toxic through oxygen radicals but no carcinogenic effect has been reported.
3. Sometimes the intensity of peroxisome proliferation and the carcinogenic effect did not correlate. Tiadenol is a strong peroxisome proliferator but only a weak carcinogen.
4. Without additional peroxidative treatment the level of malondialdehyde, a parameter for lipid peroxidation, in liver homogenates of treated rats was unchanged or even reduced.

From these findings it seems unlikely that hydrogen peroxide-induced DNA-damage alone leads to cancer after treatment with peroxisome proliferators, even though a role for this substance at a different level of the carcinogenic process cannot be ruled out. Tumorigenic mechanisms could very well exist which operate apart from active oxygen species, and even totally independently of peroxisome proliferation.

2. Expression of a special genetic programme

Peroxisome proliferators induce many different effects outside the peroxisome. There is some evidence for the presence of a specific receptor enabling peroxisome proliferators to induce the genetic expression of a special programme in the cell. Carcinogenic activity could well be related to such a programme. Two extraperoxisomal effects of peroxisome proliferators will be discussed:

1. Cytochrome P-450

Overall cytochrome P-450 is moderately increased. Almost all of this increase is due to the isoenzyme cytochrome P-452. Cytochrome P-452 is present at high concentrations in untreated animals (22% of overall cytochrome P-450). ω-Hydroxylation of lauric acid, which is almost entirely catalysed by this isoenzyme, is increased 2.5 to 7-fold after treatment.

The biological significance of this increase is unclear, because lauric acid physiologically is only present in small amounts. Possibly it can support the enhanced β-oxidation by participation in the conversion of some of the fatty acid to succinic acid. Succinic acid is further converted to oxaloacetate in the citric acid cycle which is needed to take up the increased amounts of acetyl-CoA produced during β-oxidation. Also competition between lauric acid hydroxylation and triacylglycerol synthesis is discussed. The close connection with fatty acid metabolism in general is evident. Cytochrome P-452 could also influence arachidonic acid metabolism, which produces active intermediates like prostaglandins, leukotrienes or possibly carcinogenic olefinic epoxides. There is a metabolite that is only formed by this isoenzyme, but its chemical nature has not yet been determined. The significance of changes in cytochrome P-450 for carcinogenesis is unclear, but this aspect is interesting and deserves further research (arachidonic acid, oxidative reactions, cytochrome P-450 changes in tumour promotion).

2. Carbohydrate metabolism

Carbohydrate metabolism enzymes show several changes after treatment with traditional tumour enhancing compounds like phenobarbital. The changes induced by peroxisome proliferators seem to be very similar. Therefore these changes could well be related to a tumour enhancing gene programme. They do not simply seem to lead to a "metabolic chaos".

There was a reduction of glycolytic enzymes and an increase in glucose 6-phosphate dehydrogenase and malic enzyme.

Therefore glycolysis could be reduced and some intermediates accumulate because the decreased enzymes are mainly at the end of the glycolytic chain (f. i. pyruvate kinase). These intermediates may be used for syntheses.

Glucose might be shifted away from glycolysis towards the pentose phosphate cycle reflected by the increase in G6PDH. This might increase ribose formation thus providing substrates for DNA-synthesis. Moreover a greater amount of NADPH is formed, by G6PDH as well as by malic enzyme. NADPH could also be used for DNA-synthesis and in addition it might participate in detoxification reactions providing the cell with reducing equivalents.

All this is in accordance with the observation that peroxisome proliferators are mitogenic. Peroxisome proliferators seem to induce a "new cell state" very similar to the one that has been proposed for phenobarbital. The altered cells might show a selection advantage because of enhanced proliferation- (DNA-synthesis) and detoxification capacity (NADPH). This could lead to uncontrolled proliferation perhaps followed by tumour development.
Peroxisomal Diseases

Stöckler, Sylvia

Clinical Presentation of Peroxisomal Diseases

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The clinical relationship between all the disorders assigned to peroxisomal dysfunction is variable. Zellweger syndrome and neonatal adrenoleukodystrophy are clinically closely related. Symptoms like muscular hypotonia, seizures with neonatal onset, psychomotor retardation, craniofacial dysmorphism and ocular involvement occur in both diseases. The clinical course is generally severe, but milder forms are described in both diseases. Radiological and morphological criteria have to be applied to distinguish Zellweger syndrome from neonatal adrenoleukodystrophy: adrenal atrophy and systemic infiltration of macrophages are typical for neonatal adrenoleukodystrophy; stippled skeletal calcifications and signs of cerebral and renal dysgenesis occur in Zellweger syndrome.

At present, neither clinical nor morphological criteria exist for distinguishing Zellweger syndrome clearly from hyper-pipecolic aciduria or infantile Refsum's disease.

Rhizomelic type of chondrodysplasia punctata manifest at birth with symmetrical shortening of proximal extremities, craniofacial dysmorphism, skin abnormalities and cerebral dysfunction. Radiologically stippled skeletal calcifications (chondrodysplasia punctata) can be found. The clinical course is usually severe.

The clinical course of adrenoleukodystrophy and phytanic acid storage disease (M. Refsum) is quite different from the above mentioned diseases; nevertheless there are some common clinical symptoms: the age of onset in X-linked adrenoleukodystrophy ranges from late infancy to adolescence, behavioural changes, loss of concentration, impairment of vision and hearing, atactic gait combined with signs of adrenal hypofunction (dark coloured gingiva and skin, frequent vomiting) are characteristic for the disease. Computer tomography or magnetic resonance imaging reveal pronounced occipital leukodystrophy. Death ensures 1—5 years after the first manifestation of symptoms. Refsum's disease manifests from early childhood until the 5th decade with retinitis pigmentosa, peripheral neuropathy, cerebellar ataxia and evolution of cerebrospinal fluid protein. Nerve deafness, skeletal, skin and ECG abnormalities occur less frequently. A phytanic acid-free diet can bring about significant improvement of the clinical symptoms.

No clinical relationship between hyperoxaluria I and acatalasaemia and the above mentioned diseases exist. Hyperoxaluria I is characterized by renal and extrarenal oxalate deposits. Renal insufficiency predominates the clinical course. Cases with early infantile onset and rapid renal deterioration have been described. Patients with acatalasaemia suffer from painful ulcers in the mouth; otherwise no further clinical symptoms are found.

The common trait of all these diseases is an impairment of biochemically defined peroxisomal functions. Accordingly diseases with global peroxisomal dysfunction (Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum's disease, hyper-pipecolic aciduria), diseases with more than one peroxisomal dysfunction (rhizomelic type of chondrodysplasia punctata, Zellweger variants) and diseases with one single peroxisomal dysfunction (adrenoleukodystrophy, Refsum's disease, hyperoxaluria I, acatalasaemia) are classified.

The clinician's search for peroxisomal diseases will give more insight in the frequency and the clinical variations of these disorders.

With the exception of Refsum's disease, the therapeutic possibilities at the present state of knowledge are poor. Prenatal diagnosis of most of these diseases can be offered to affected families.

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Pathology of Peroxisomal Disorders

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Peroxisomal disorders are generally classified into two broad groups:
1) those with more than one peroxisomal defect often with structurally abnormal peroxisomes (generalized) and
2) those with an abnormality in a single peroxisomal enzyme and usually with morphologically intact peroxisomes. An abnormality in the number or size of...
Peroxisomes is observed in cerebro-hepato-renal (Zellweger) syndrome, infantile Refsum disease and neonatal adrenoleukodystrophy. Peroxisomes are usually morphologically intact and particulate in pseudo-neonatal adrenoleukodystrophy, adrenoleukodystrophy, its adrenomyeloneuropathic variant, adult Refsum disease and thiolase deficiency. Recently described Leber's disease with hepatomegaly also is reported to be associated with a lack of peroxisomes. The generalized peroxisomal disorders generally manifest themselves in infancy by the presence of seizures, psychomotor retardation and dysmorphic features.

The prototypic generalized peroxisomal disorder, cerebro-hepato-renal (Zellweger) syndrome, is characterized principally by stippled calcifications (especially of patella, femur, humerus), micronodular cirrhosis or fibrosis, renal cortical microcysts of tubular and glomerular origins, cataracts, abnormal central white matter, abnormal neuronal migrations, particularly in the form of centrosylvian pachygyria and polymicrogyria and neuroglial lipidosis.

Infantile Refsum's disease is characterized by hepatomegaly with large numbers of PAS-positive striated, birefringent macrophages with angulate lysosomes containing trilaminar structures.

Hyperpipecolic acidemia also exhibits hepatomegaly with micronodular cirrhosis and hepatocytic glycolipid inclusions, PAS-positive macrophages in adrenals, demyelination and astrocytosis of central white matter and PAS-positive macrophages in the CNS.

Rhizomelic chondrodysplasia punctata, which displays morphologically intact peroxisomes, is characterized primarily by growth retardation due to the shortening of long bones, widespread calcifications, joint contractures, cataracts and optic atrophy.

Neonatal adrenoleukodystrophy displays hepatic fibrosis, adrenocortical atrophy, PAS-positive striated macrophages containing angulate lysosomes in liver, thymus, spleen, lymph nodes, gastro-intestinal tract and adrenals; and demyelination and astrocytosis of central white matter of moderate inflammatory intensity (particularly occipital lobe and cerebellum). Abnormal migrations in the form of polymicrogyria, cerebral and cerebellar heterotopias and dysplastic olives may exist.

Although adrenoleukodystrophy is the best described and most frequent peroxisomal disease with morphologically intact peroxisomes, it differs greatly from its colleagues: pseudo neonatal adrenoleukodystrophy (n = 1), thiolase deficiency (n = 1) and adult Refsum disease, and differs somewhat from its adrenomyelo neuropathic variant. Leber's disease with hepatome-
Galy (n = 1 or 2) is tentatively segregated from both groups pathologically, in spite of the reported absence of peroxisomes, until a more extensive pathologic profile with confirmation of the peroxisomal deficiency is available. Pseudo neonatal adrenoleukodystrophy is characterized by hepatomegaly with PAS-positive macrophages (also in adrenals, thymus and lymph nodes), adrenocortical atrophy with striated cells, demyelination of CNS white matter (occipital and cerebellar) and mild abnormal neuronal migrations of cerebrum and cerebellum, persistence of Cajal-Retzius cells, focal olivary dysplasia and dorso-medial displacement of Clarke's nucleus. Adreno-leukodystrophy shows prominent adrenocortical atrophy with striated cells and severe inflammatory demyelinating CNS lesions.

Lamellar structures have been identified in adrenocortical, Leyding, Schwann and, probably, oligodendrocytic cells. The adreno-myeloneuropathic variants display the same adrenal lesion but, being adults, also may have testicular atrophy and inconstant CNS demyelination; its predominant neuropathologic abnormality is that of degeneration of ascending and descending spinal tracts. Adult Refsum disease also exhibits degeneration of CNS tracts (brainstem) and neuronal loss, but its major lesions are those of pigmented retinopathy and hypertrophic polynephropathy. One patient with thiolase deficiency displays mild hepatic fibrosis, renal cortical microcysts, adrenocortical atrophy with striated cells and CNS demyelination and astrocytosis, predominantly non-inflammatory. Abnormalities of neuronal migration are mild, consisting of subcortical Purkinje cells and probable focal polymicrogyria. Leber disease with hepatomegaly is reported to show optic disc swelling, mild hepato-megaly and renal microcysts, if one accepts the case of Dekaban (1969) as a bona fide example.

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Biochemical Investigations in Peroxisomal Disorders

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The peroxisomal disorders represent a newly recognized group of inherited diseases caused by an impairment in one or more peroxisomal functions. The cerebro-hepato-renal syndrome of Zellweger is generally considered to be the prototype of this group of disorders (see l.c. (1) for review). The peroxisomal disorders can be classified into three groups (A, B and C) based upon the generalized (A), multiple (B) or single (C) loss of peroxisomal functions.

In group A comprised of Zellweger syndrome, infantile Refsum disease, neonatal adrenoleukodystrophy and hyper-pipecolic acidemia, there is a generalized loss of peroxisomal functions due to a strong deficiency of peroxisomes. In body fluids from affected patients there is an accumulation of very long chain fatty acids, the bile acid intermediates di- and trihydroxycholestanolic acid, pipecolic acid and phytanic acid. In these patients accumulation of very long chain fatty acids and bile acid intermediates results from an impairment in the peroxisomal ß-oxidation of these compounds due to a deficiency of the peroxisomal ß-oxidation enzyme proteins. We recently discovered that the first step in the degradation of L-pipecolic acid is catalysed by an H2O2-generating oxidase present in peroxisomes in man but not present in rat. Furthermore, we found that the activity of this enzyme L-pipecolic acid oxidase was strongly deficient in livers from Zellweger patients (controls: 24.8 ± 5.7 mg 7) versus Zellweger patients: 0.26 ± 0.19 pmol/min · mg (4)) thus explaining the accumulation of L-pipecolic acid in patients from group A (2).

Group B is comprised of rhizomelic chondrodysplasia punctata and Zellweger-like syndrome (see l.c. (1) for review). In the rhizomelic form of chondrodysplasia punctata there is a deficiency of dihydroxyacetone-phosphate acyltransferase, alkyl dihydroxyacetone-phosphate synthase and phytanic acid oxidase. Furthermore, it was recently discovered in our own laboratory (3) and Dr. Hugo Moser's laboratory (4) that the peroxisomal 3-oxoacyl-CoA thiolase enzyme protein is not present in the mature form but in the precursor form (M, 44 · 105).

Group C is comprised of X-linked adrenoleukodystrophy, pseudo-Zellweger syndrome (peroxisomal thiolase deficiency), pseudo-neonatal adrenoleukodystrophy (acyl-CoA oxidase deficiency), hyperoxaluria type I (alanine glyoxylate aminotransferase deficiency), classical Refsum disease (phytanic acid oxidase deficiency) and acatalasemia. Based upon the finding that the peroxisomal oxidation of lignoceryl-CoA (but not of lignoceric acid) was normal in X-linked adrenoleukodystrophy fibroblasts, it has been suggested that the defect in X-linked adrenoleukodystrophy is at the level of a deficient activation of very long chain fatty acids to their CoA-esters (5). In order to investigate this possibility we have developed a method which allows the isolation of micro-peroxisomes from cultured fibroblasts and measured the
very long chain fatty acid activating enzyme activity in peroxisomal fractions isolated from control and X-linked adrenoleukodystrophy fibroblasts (6). The activity of hexacosanoyl-CoA synthetase in peroxisomal fractions of control and X-linked adrenoleukodystrophy fibroblasts was found to be $63.4 \pm 8.4$ (5) and $12.8 \pm 4.3$ (5) pmol/min · mg, respectively, indicating that X-linked adrenoleukodystrophy is, indeed, due to a deficient ability of peroxisomes to activate very long chain fatty acids (6).

Recent studies in different laboratories have shed new light on the primary defect in the peroxisomal disorders characterized by a deficiency of peroxisomes. Firstly, it has become clear from genetic complementation studies carried out in Graz and Amsterdam (7) and Amsterdam (8) that there is genetic heterogeneity within Zellweger syndrome, infantile Refsum disease, neonatal adrenoleukodystrophy and hyper-pipecolic acidemia, indicating the involvement of different genetic mutations. Secondly, available evidence suggests that the genetic mutations in Zellweger syndrome and the other peroxisome deficiency disorders affect the uptake machinery of peroxisomes responsible for the import of peroxisomal enzyme proteins into peroxisomes. This conclusion stems from the fact that the integral membrane proteins are not only normally present in Zellweger patients (9) but also contained within a membrane (peroxisomal ghosts) as shown by immunofluorescence studies in fibroblasts from Zellweger patients (10, 11) using antisera directed against integral membrane proteins. In summary, much has been learned over the past few years about peroxisomes and peroxisomal disorders.

This has led to the development of methods allowing postnatal as well as prenatal detection of each of the diseases known so far (for discussion see l. c. (1)).

References


Roscher, A., S. Höfler, G. Höfler, E. Paschke, F. Paltauf, A. Moser and H. Moser

Genetic Complementation Analysis in Peroxisomal Disorders

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The cerebro-hepato-renal (Zellweger) syndrome, the infantile form of Refsum’s disease, the neonatal form of adrenoleukodystrophy and hyper-pipecolic acidemia represent peroxisomal polydysplasia syndromes with apparently overlapping clinical, biochemical and histological features. The question therefore arose as to whether these syndroms are merely variants of the cerebro-hepato-renal syndrome or idiopathic disease entities distinct from cerebro-hepato-renal syndrome.

Among other biochemical disturbances these diseases exhibit an impairment of the peroxisomal steps of plasmalogen biosynthesis. By using a sensitive double-label, double-substrate incubation technique (1) we recently demonstrated that this disturbance is less severe in fibroblasts from the neonatal form of adrenoleukodystrophy as compared to cerebro-hepato-renal syndrome cells (2).

The question of genetic heterogeneity was approached by complementation analysis using somatic cell hybridization. This requires the bringing together of the defective gene of each mutant into the same cell by cell fusion and testing for restoration of function. Correction of defective peroxisomal steps of plasmalogen biosynthesis was tested in fused cell populations of the neonatal form of adrenoleukodystrophy, cerebro-hepato-renal syndrome and control fibroblasts. Cells were seeded at a 1:1 ratio of each parental cell-line, fused with 700 g/l polyethylene glycol 4000 containing a volume fraction of 0.16 dimethylsulphoxide...
and subsequently applied to a Ficoll stepped density gradient at 1 g (heterokaryon enrichment procedure). Two fractions, containing either only unfused cells ("cocultivation control") or predominantly (70—80%) multinucleated cells, were collected and further cultivated in the presence of $[^{14}C]$hexadecanol and $[^{3}H]$hexadecylglycerol for analysis of peroxisomal steps of plasmalogen biosynthesis.

In fusions of

cerebro-hepato-renal syndrome × control,
neonatal form of adrenoleukodystrophy × control and
cerebro-hepato-renal syndrome × neonatal form of adrenoleukodystrophy
pathological values were obtained in "cocultivation control" fractions.

These values reverted to normal in enriched fused cells. In analysing a larger series of cell lines, clearly different complementation groups were defined in some patients, which cannot be distinguished by existing criteria (cf. table).

The results are consistent with the hypothesis that at least 4 different gene mutations may cause phenotypes clinically described as neonatal form of adrenoleukodystrophy and/or cerebro-hepato-renal syndrome.

References


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Aubourg, P. and S. Guidoux

Large Scale Physical Mapping Around the Adrenoleukodystrophy Gene: A First Step Toward the Isolation of Adrenoleukodystrophy Gene

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Recent data suggest that the primary defect of X-linked adrenoleukodystrophy involves the formation of the acyl-CoA derivative of very long chain fatty acids and possibly a specific peroxisomal very long chain fatty acids ligase deficiency. However, this enzyme has not yet been purified and as for many other genetic disorders for whom the gene product is still unknown, the isolation of the adrenoleukodystrophy gene depends first on its localization on the human genome. Adrenoleukodystrophy gene has been assigned to the distal portion of the long arm of the X-chromosome called the Xq2—8 region: linkage analyses have shown that the adrenoleukodystrophy gene is tightly linked to glucose-6-phosphate dehydrogenase and to an anonymous recombinant DNA probe (St14). Only one recombination has been found between adrenoleukodystrophy and St14 in more than 100 meiosis, permitting an estimate of the genetic distance between them of less than 1 centi-Morgan (cM). The St14 probe, which shows a Taq1 or Msp1 restriction fragment length polymorphism (RFLP) in the Xq2—8 region, is very useful, since more than 90% of females are informative for this probe. Therefore, heterozygote status for adrenoleukodystrophy can be determined by the combination of very long chain fatty acids assay (plasma, fibroblasts) and St14 linkage analysis in more than 99% of females at risk.

More than 10 genes have been mapped to Xq2—8, including coagulation factor VIII, Emery Dreyfuss myopathy, glucose-6-phosphate dehydrogenase and red-green colour pigments. Until now, no adrenoleukodystrophy or adrenomyeloneuropathy patients have been found to have haemophilia A, Emery Dreyfuss myopathy or glucose-6-phosphate dehydrogenase deficiency. However, approximately 50% of adrenoleukodystrophy/adrenomyeloneuropathy patients have various degrees of red-green colour discrimination defects (i.e. they are dichromatic with protanopic or deuteranopic phenotype) as compared with...
pigment genes have been cloned, the exact localization adrenoleukodystrophy of very long chain fatty acids. An excess is related in some way to pathogenesis of very long chain fatty acid metabolism in a patient with Refsum disease (12). In that study, a patient with Refsum disease was also administered deuterated water by mouth, and it was found that there was no significant labeling of plasma.

Pathogenesis of very long chain fatty acid accumulation

The work of Singh et al. (2) and the recent studies of Hasmi et al. (3) and Wanders et al. ((4)) provide firm evidence that the basic defect in adrenoleukodystrophy is the impaired capacity to degrade very long chain fatty acids, a reaction that takes place in the peroxisome (5).

The very long chain fatty acids that accumulate in adrenoleukodystrophy arise both from endogenous synthesis and from the diet. Endogenous synthesis utilizes the microsomal fatty acid elongating system (6). As will be emphasized again in the discussion of therapy it appears that a single enzyme elongates saturated and monounsaturated fatty acids (7). Tsuji et al. (6) have reported that the microsomal fatty acid elongating system in X-linked adrenoleukodystrophy cultured skin fibroblasts is more active than in control cells, thus suggesting that there is overproduction of very long chain fatty acids. The physiological significance of this finding is difficult to assess.

The evidence for a dietary origin of very long chain fatty acids has been provided by the in vivo studies of Kishimoto et al. (8). These investigators administered deuterium labeled hexacosanoic acid (C 26:0) by nasogastric tube to a terminally ill adrenoleukodystrophy patient for the last 100 days of his life. Post mortem analysis of the cholesterol ester fraction in an actively demyelinating zone showed that up to 90% of the C 26:0 contained the deuterium label. This finding indicates that in this portion of brain 90% of C 26:0 was derived from the diet during the last 100 days of the patient's life.

While this result is a striking demonstration of the dietary contribution to very long chain fatty acid accumulation, there is indubitably evidence that endogenous synthesis is an additional source, and, in fact, it now appears that endogenous synthesis is quantitatively more important. Tsuji et al. (6) and Rizzo et al. (9) have demonstrated that human cultured skin fibroblasts synthesize C 26:0. We have shown that after an adrenoleukodystrophy patient was administered deuterated water by mouth, there was substantial incorporation of label into the C 26:0 of plasma and post mortem tissues (10, 11). This result differs markedly from an analogous study of phytanic acid metabolism in a patient with Refsum disease (12). In that study, a patient with Refsum disease was also administered deuterated water by mouth, and it was found that there was no significant labeling of plasma.
phytanic acid. This and other studies indicate that phytanic acid is of dietary origin exclusively. Results of dietary manipulations are consistent with these findings: In Refsum disease patients dietary phytanic acid restriction alone will normalize plasma phytanic acid levels (13). As will be discussed later, dietary C 26:0 restriction alone does not alter plasma C 26:0 levels in adrenoleukodystrophy patients. In order to achieve such a reduction the dietary restriction must be combined with measures that reduce endogenous synthesis.

How do excess very long chain fatty acids lead to tissue damage?

1) Excess very long chain fatty acids increase membrane microviscosity

Knazek et al. (14) have shown that membrane microviscosity is increased in erythrocytes of adrenoleukodystrophy patients. Recently this same group reported that cultured adrenocortical cells showed diminished corticotropin stimulated cortisol production when very long chain fatty acids were added to the culture medium at a concentration equivalent to that found in adrenoleukodystrophy plasma (15). Meyer et al. (16) have reported (16) that, unlike mononuclear leukocytes from unaffected individuals, leukocytes from a patient with adrenoleukodystrophy had no demonstrable corticotropin binding sites. These provocative findings suggest that very long chain fatty acid excess is toxic because it increases membrane viscosity, and in this way suppresses the availability of hormone receptors.

This mechanism could account, at least in part, for the adrenal and testicular insufficiency in adrenoleukodystrophy, and in an as yet undefined manner, for the neuropathology.

2) Additional, possibly immunological pathogenetic mechanisms

Unlike most other “storage” diseases, in adrenoleukodystrophy there is a striking variability in phenotype among members of the same kindred. Within the same kindred, males with the adrenoleukodystrophy genotype may present either with the fatal form of childhood adrenoleukodystrophy, with the milder adult adrenomyeloneuropathy, with adrenal insufficiency without neurological involvement, and occasionally without apparent nervous system or adrenal involvement (17, 18). There is no correlation between plasma very long chain fatty acid levels and the severity of the neurological manifestation of adrenoleukodystrophy (17, 18). This has led us to propose that the very long chain fatty acid abnormality is “necessary but not sufficient” for the pathogenesis of the neuropathology; and that additional pathogenetic mechanisms play a role. For reasons discussed elsewhere in more detail (17—19) the present hypothesis is that immunopathological mechanisms are involved. This hypothesis is based upon

1) the perivascular cuffs of lymphocytes and monocytes which are a characteristic feature in adrenoleukodystrophy brain white matter,

2) the immunological typing of these cells, which shows a pattern consistent with an immunological response to an antigen within the nervous system (19),

3) increased levels of immunoglobulins within the nervous system (20), and

4) the presence of complement fixing antibodies to central nervous system myelin in the serum of adrenoleukodystrophy patients (Lee Koski & Hugo W. Moser — unpublished observations). It is our working hypothesis that the basic enzyme defect in adrenoleukodystrophy causes an alteration of fatty acid composition, and that this leads to an autoimmune response in some, but not in all, persons with the adrenoleukodystrophy genotype.

Therapy

General comments

The current approach to the therapy of adrenoleukodystrophy is based upon the hope that it will be possible to achieve results analogous to the therapy of Refsum disease. It is clearly established that dietary restriction of phytanic acid can restore cardiac and peripheral nerve function, and arrest progression of visual system defects (13). The dietary management of adrenoleukodystrophy is more complex than that of Refsum disease. As already noted very long chain fatty acids are derived from endogenous synthesis as well as dietary sources. Dietary restriction of very long chain fatty acids alone fails to alter plasma very long chain fatty acids (21, 22). However, partial normalization of plasma and red blood cell very long chain fatty acid levels is achieved when dietary very long chain fatty acid restriction is combined with measures that reduce the rate of synthesis of saturated very long chain fatty acids. Such favourable biochemical results have now been documented in more than 40 patients (9, 17, 18). In order to diminish the rate of synthesis of saturated very long chain fatty acids these patients were administered a glycerol trioleate oil. It appears that oleic acid (C 18:1) is chain elongated by the same enzymes that elongate C 16:0 or C 18:0 fatty acids, and that in this way the production of saturated very long chain fatty acids is reduced competitively. The capacity to manipulate the plasma
saturated very long chain fatty acid levels in adrenoleukodystrophy patients is an important advance. We are now conducting a randomized prospective study to determine whether partial normalization of these levels has a favourable effect on the clinical course. This involves 30 men with adrenomyeloneuropathy and up to 20 women, who are heterozygous for adrenoleukodystrophy and have neurological disability attributable to this condition. We expect to complete this study by the end of 1989.

If dietary therapy does have a favourable effect on the neurological manifestation of adrenoleukodystrophy, then this approach, or modification thereof would probably represent the major therapeutic strategy. Other approaches are also under investigation, but since they have more serious side effects, their use is at present restricted to special circumstances. These approaches are bone marrow transplantation (10, 11) and immunosuppression (23). Bone marrow transplantation is a particularly interesting challenge. It has already been shown that enzymatically competent bone marrow cells can be transplanted, and that such a transplant results in a reduction of plasma very long chain fatty acid levels (10, 11). If such a procedure can be shown to produce clinical benefit, it would open up the possibility of gene replacement therapy, if and when such an approach becomes generally safe and feasible.

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