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Establishment of a normal range of morphometric values for peroxisomes in paediatric liver

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Abstract. The size and number of hepatic peroxisomes was investigated in 16 control paediatric liver biopsies from patients ranging in age from 3 months to 18 years one fetal liver specimen and one paediatric autopsy liver. The area, diameter, volume density (V_v), numerical density (N_v) and surface density (S_v) of the peroxisomes was recorded using randomly selected electron micrographs. The mean diameter of peroxisomes in control paediatric liver was 0.56 μm, the mean V_v was 1.67%, the mean N_v was 0.125 per μm^{+3} and the mean S_v was 0.161 per μm. No correlation was found between the size and number of hepatic peroxisomes and the age or sex of the patient. Peroxisomes in the fetal liver were smaller than those in biopsy tissue and had a mean diameter of 0.42 μm. Peroxisomes were identified in autopsy tissue and were enlarged with a mean diameter of 0.75 μm, most probably due to post-mortem swelling. A range of morphometric values in paediatric liver has now been established.

Key words: Peroxisomes – Morphometry – Liver

Introduction

Peroxisomes are important organelles which are most numerous in mammalian liver and are involved in many metabolic functions (Hruban et al. 1972). Their importance in cellular metabolism has been emphasised by the discovery of inherited diseases such as Zellweger syndrome, neonatal adrenoleukodystrophy and infantile Refsum's disease, where many peroxisomal functions are deficient, leading to severe clinical consequences with most patients not surviving the first year (Schutgens et al. 1986; Moser et al. 1991). Hepatic peroxisomes in these patients show alterations in their morphological features: usually a reduction or increase in their size and number (Roels et al. 1988, 1991; Hughes et al. 1990; Roels 1991). Morphometric data on peroxisomes in various peroxisomal disorders are now appearing in the literature [Roels et al. 1988, 1991; Hughes et al. 1990, 1992, 1993 (data to be published); De Craemer et al. 1991a, b]. Some data on control patients have been published (Table 1). However much of these data relate to adult patients and there is a need to compare liver from peroxisomal disorder patients who are usually aged from several weeks or months to a few years, with aged matched controls.

The purpose of this study was to establish a range of normal morphometric values for peroxisomes in paediatric liver with which to compare liver tissues from patients with peroxisomal disorders and also other disease states which may have some peroxisomal involvement.

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Materials and methods

Liver biopsies which had been processed for routine electron microscopic studies were examined in a retrospective study of peroxisome morphology. Sixteen controls were chosen from biopsies which were described as normal by histological criteria. The patients ranged in age from 3 months to 18 years and were undergoing investigations for various conditions not related to peroxisomal disorders. We also examined autopsy liver from a 4-month-old boy where the postmortem interval was approximately 8–9 h. Autopsy liver from a fetus aged 12 weeks was also included.

Liver was obtained by needle biopsy or at autopsy and fixed immediately by immersion in a mixture of 4% formaldehyde and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.3. After fixation for 2 h at room temperature the tissue was washed twice in 0.1 M sodium cacodylate buffer, postfixed in 1% osmium tetroxide, washed in cacodylate buffer again and stained en bloc with 2% aqueous uranyl acetate. The tissue was then dehydrated in a graded series of ethanol and embedded in Spurr's low-viscosity epoxy resin. Polymerisation was carried out at 70° C under vacuum for at least 12 h. Silver-gold thin sections were cut on a Reichert Ultracut microtome, stained with uranyl acetate for 3 min and lead citrate for 3 min, and examined in an Hitachi 7000 electron microscope.

Twenty electron micrographs of each section of liver were randomly selected in relation to the grid bars at a magnification of × 10000 and enlarged photographically to × 22000. No distinction

Table 1. Morphometric values for control liver which appear in the literature

Author	Sample	Mean diameter (μm)* (range)	Volume density (%)	Numerical density (per μm³)	Surface density (per μm)
DeCraemer et al. 1991b	Six adults	0.529	1.06	0.096	0.108
	One 6-week-old infant	0.445	0.70	0.128	0.085
	One 4-month-old infant	0.529	1.18	0.110	0.131
DeCraemer et al. 1991a	Eight controls	0.517 (0.445–0.620)	1.02 (0.71–1.41)	0.100 (0.052–0.131)	0.107 (0.083–0.149)
Koch et al. 1978	Four adults	–	0.97	–	0.07
Jezequel et al. 1983	Not stated	–	0.97	–	0.072
Poll-The et al. 1988	One adult	0.534	–	–	
	One baby	0.478	–	–	
Roels et al. 1988	One adult	0.534	–	–	
	One 5-week-old female	0.478	–	–	
	One 4-month-old male	0.481	–	–	
Roessner et al. 1978	Fourteen adults	–	0.99	–	–
Rohr et al. 1976	Four adults	–	1.2	–	–
Sternlieb and Quintana 1977	Four (12–20 years old)	0.618 (0.25–1.34)	–	–	
This study	Sixteen paediatric (3 months–18 years)	0.56 (0.51–0.63)	1.67 (0.7–3.2)	0.125 (0.057–0.188)	0.161 (0.073–0.262)

* d-circle, not corrected for section thickness

was made between periportal and centrilobular hepatocytes. A carbon grating replica with 2160 lines per millimetre (Probing and Structure) was photographed and enlarged at the same magnification as each batch of micrographs and the magnification factor calculated. The area, perimeter and diameter of the peroxisomal profiles, and the area of the hepatocyte cytoplasm were measured by manual tracing on a digitiser linked to a computer using a Video Trace software package (Leading Edge Technology, Adelaide, Australia). The diameter was recorded as the d-circle which is the diameter of the circle having the same area as the measured organelle. The volume density (V_v), numerical density (N_v), and surface density (S_v) were calculated using the methods of Weibel (1979). The V_v is a measure of the total volume of the peroxisomes as related to the total hepatocyte volume (including the hepatocyte cytoplasm and nucleus). This was calculated by dividing the sum of the area of all the peroxisomal profiles by the sum of the area of the hepatocyte cytoplasm and nucleus and was expressed as the relative V_v (%).

The N_v is the number of peroxisomes per unit cell volume and was calculated using the formula of Weibel (1979):

$$N_v = \frac{1}{\beta} \cdot \frac{(N_A)^{3/2}}{(V_v)^{1/2}} \cdot k$$

where k is the size distribution coefficient, β is the shape related coefficient as determined according to Weibel (1979), and N_A is the ratio between the number of peroxisomes and the total area of the hepatocytes.

The S_v is the total membrane area of the peroxisomes expressed per cellular volume and was calculated from the formula:

$$S_v = \frac{4B_A}{\pi}$$

where B_A is the ratio between the sum of the peroxisome perimeters and the total area of the hepatocytes.

The results were not corrected for section thickness, since the sections used were all of similar thickness according to the interference colours on the ultramicrotome during sectioning.

Results

Peroxisomes were readily identified in liver biopsies as single membrane bounded organelles with a homogeneous moderately electron-dense matrix (Fig. 1a). Electron-dense or crystalline cores were not normally present. Section profiles of the peroxisomes were roughly circular and they ranged in diameter from 0.11 to 1.11 μm. A frequency histogram of the diameter of peroxisomes is shown in Fig. 2. The V_v of peroxisomes in the 16 paediatric controls ranged from 0.7% to 3.2% with a mean of 1.67%. The N_v of peroxisomes ranged from 0.057 to 0.188 per μm³ with a mean of 0.125 per μm³ and the surface density of peroxisomes ranged from 0.073 to 0.262 per μm with a mean of 0.161 per μm.

Regression analysis showed that there was no correlation between the age of the patient and the mean area of peroxisomes ($R^2 = 0.1\%$).

In the fetal specimen the peroxisomes could easily be recognised and were slightly smaller in mean area than in biopsy livers (Fig. 1b, Table 2). In the autopsy liver the peroxisomes were larger than in the biopsy and fetal livers and had a flocculent, extracted appearance (Fig. 1c). Profiles of smooth endoplasmic reticulum were often flattened and condensed along the peroxisomal membrane in autopsy tissue (Fig. 1c).

The mean area of peroxisomes, and the mean diameter, the volume, numerical and surface densities in 18 control liver specimens are shown in Table 2.

Discussion

The diameter of peroxisomes in the control liver biopsies in this study is similar to those which already appear in

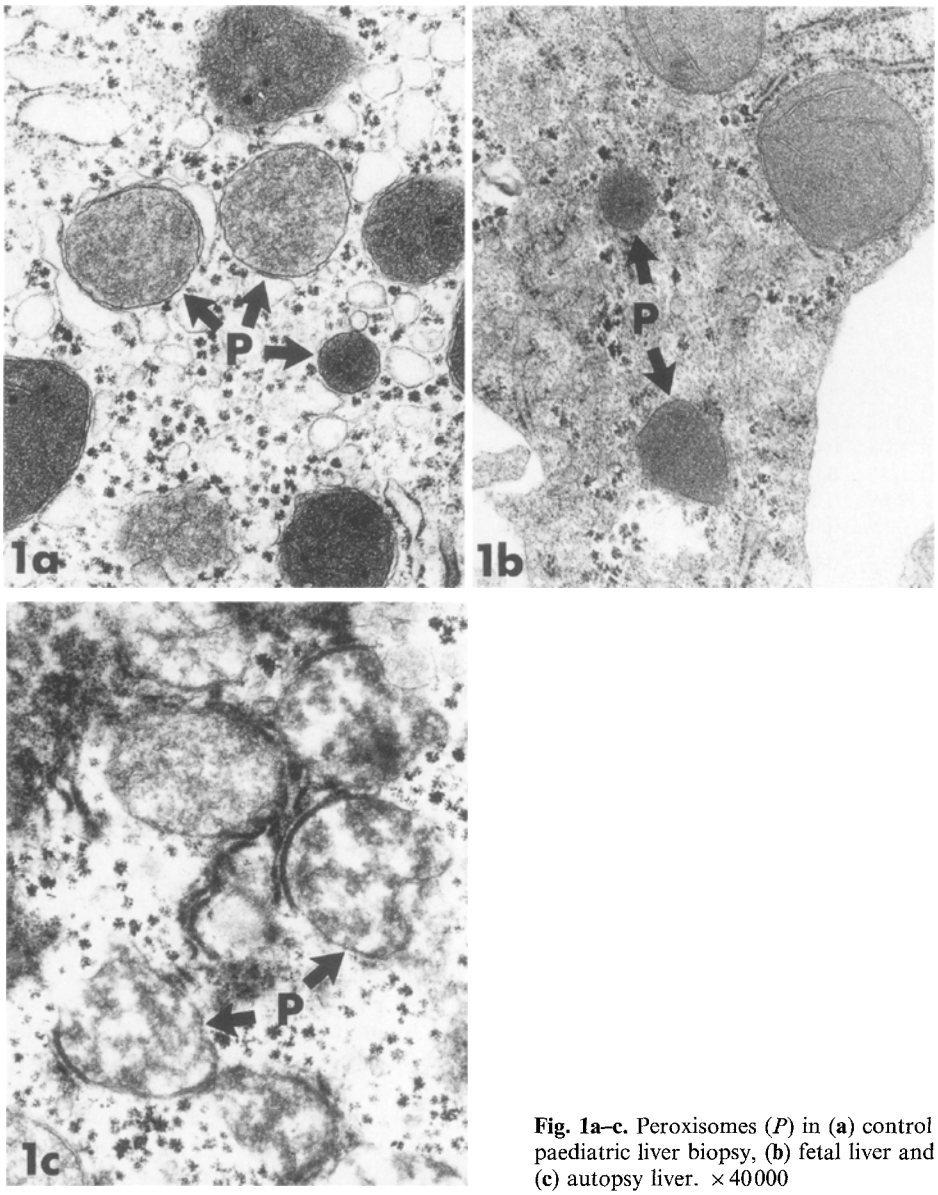

Fig. 1a–c. Peroxisomes (*P*) in (**a**) control paediatric liver biopsy, (**b**) fetal liver and (**c**) autopsy liver. ×40000

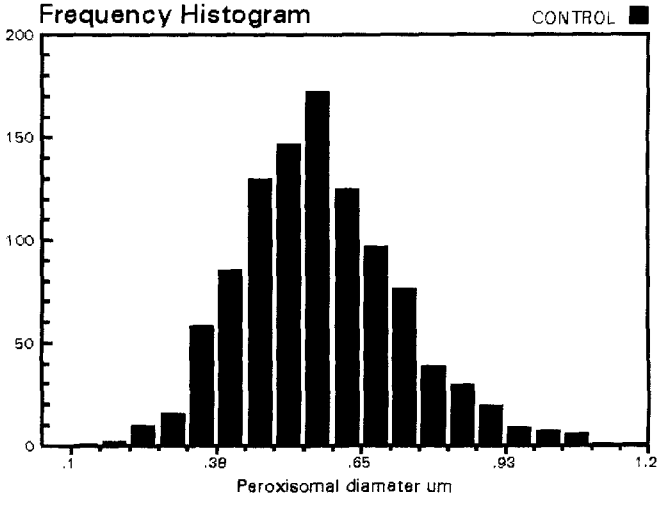

Fig. 2. Frequency histogram of peroxisomal diameter in control paediatric liver biopsies

the literature (Table 1). In particular there appears to be little difference in the mean diameter of peroxisomes in liver from adult patients and the liver of paediatric patients. In two infants aged 5 weeks and 4 months, Roels et al. (1988) found peroxisomes with a mean diameter slightly less than that of the adults. De Craemer et al. (1991a, 1991b) also reported slightly smaller peroxisomes in one of two infant biopsies. This patient [and one of Roels et al. (1988)] was only 5 weeks old. We did not have any patients this young in our control group.

The relative V_v, N_v and S_v reported in this study are somewhat higher than the data reported in the literature. This may be because our control tissues are from infants and children, compared with adult liver which has been reported in most studies. It is also possible that some of the control patients in this study had been treated with drugs, or suffered other conditions, which may have caused an increase in the number of peroxisomes. Unfortunately it is not possible to obtain liver biopsies from

Table 2. Morphometry of peroxisomes in control paediatric liver

Case	Age	*n*	Mean area (μm^2)	SD	Area range (μm^2)	Mean diameter (d-circle) (μm)	Volume density (%)	Numerical density (per μm^3)	Surface density (per μm)
1	3 months	67	0.22	0.09	0.07–0.52	0.56	1.7	0.128	0.165
2	4 months	77	0.21	0.12	0.05–0.64	0.56	2.0	0.152	0.196
3	6 months	93	0.29	0.17	0.008–0.77	0.63	3.2	0.154	0.262
4	7 months	54	0.17	0.08	0.002–0.37	0.51	1.1	0.122	0.125
5	9 months	31	0.19	0.08	0.08–0.40	0.53	0.7	0.064	0.073
6	9 months	58	0.18	0.08	0.03–0.34	0.53	1.1	0.112	0.122
7	21 months	80	0.21	0.14	0.001–0.56	0.55	2.1	0.163	0.194
8	30 months	27	0.22	0.09	0.006–0.36	0.59	0.8	0.057	0.076
9	42 months	54	0.21	0.11	0.003–0.44	0.56	1.3	0.100	0.126
10	5 years	56	0.23	0.09	0.06–0.44	0.58	1.4	0.098	0.135
11	8 years	80	0.24	0.12	0.008–0.51	0.58	2.3	0.148	0.212
12	9 years	104	0.22	0.09	0.06–0.47	0.58	2.6	0.188	0.252
13	11 years	86	0.21	0.08	0.006–0.46	0.53	1.9	0.160	0.196
14	13 years	58	0.24	0.15	0.004–0.96	0.62	1.6	0.103	0.149
15	13 years	55	0.20	0.09	0.07–0.46	0.54	1.4	0.117	0.138
16	18 years	71	0.20	0.09	0.02–0.43	0.54	1.5	0.133	0.158
Mean	–	60	0.22	–	0.001–0.96	0.56	1.67	0.125	0.161
17[a]	12 weeks	51	0.11	0.04	0.03–0.23	0.42	0.63	0.128	0.088
18[b]	4 months	39	0.37	0.14	0.14–0.77	0.75	1.6	0.056	0.126

[a] Fetal
[b] Autopsy

genuinely "normal" infants. However, it is important to establish a range of morphometric values from these paediatric patients with which to compare liver biopsies from peroxisomal disorder patients.

The identification of peroxisomes in this study was based on purely morphological features. We considered using tissue which had been cytochemically stained for catalase to identify peroxisomes (Novikoff and Goldfischer 1969; Fahimi 1975; Roels et al. 1975), as a proportion of the smaller peroxisomes may be missed by using morphology alone (Beier and Fahimi 1986). However, it would not be possible to use retrospective liver biopsies if this was the case, as all our liver biopsies have been processed using standard electron microscopic techniques. Also in biopsies from patients with peroxisomal disorders abnormal peroxisomes need to be identified. Often these peroxisomes have low levels of catalase [Roels et al. 1986; Hughes et al. 1992, 1993 (data to be published)] and the enzyme cytochemical technique may not detect them. Beier and Fahimi (1992) have recently reported the use of immunocytochemical techniques in conjunction with automatic image analysis for identifying peroxisomes in rat liver. This technique is also unsuitable for human liver biopsies as special fixation and processing is required. The figures obtained in this study, where peroxisome identification relied on morphology only, are actually greater than most of those previously reported. We therefore feel that the use of retrospective tissue which has been routinely processed for electron microscopy has not had a significant effect on the values of V_v, N_v and S_v.

The mean peroxisomal diameter in fetal liver was smaller than that found in biopsy liver. The V_v, N_v and S_v of peroxisomes in the fetal specimen in this study were at the lower end of the range for biopsy liver. In human fetal kidney, the mean size of peroxisomes was less than that of adults, and there was little change during the 10th–18th weeks of gestation (Briére 1986). This suggests that a significant increase in the diameter of peroxisomes must take place after the 18th week of gestation or after birth to reach the level of the adult. Similarly in human fetal liver an increase in peroxisomal size takes place during development (Kerckaert 1990, as quoted in Espeel et al. 1990 and Roels 1991). Espeel et al. 1990 found an increase in the size of hepatic peroxisomes between the 7th and 18th gestational weeks. The number of peroxisomes has been reported to be higher in younger fetuses (8–10 weeks) and then falls abruptly from 12 weeks onwards (Kerckaert 1990, as quoted in Roels 1991).

In rat liver the volume of individual hepatocytes increases with age and variations occur in different organelles (Schmucker 1990). The data concerning peroxisomes are very limited and somewhat inconsistent. However, it appears that ageing has little effect on this organelle in rat liver, but during fetal and postnatal development some changes do occur. In mouse hepatocytes the V_v of peroxisomes increases 2- to 3-fold between birth and 10 days of age (Kanamura et al. 1990) and in rat hepatocytes the size and number of peroxisomes increases gradually during fetal development (Stefanini 1985). Also in the liver of baby pigs the number of peroxisomes doubles from 1 to 4 weeks of age (Giesecke et al. 1987). Our data show there is no correlation between the size or number of hepatic peroxisomes and the age of the patient. However, since our youngest patient was 3 months old, it is possible that changes may occur from birth to 3 months.

The mean diameter of the peroxisomes in the autopsy tissue was considerably enlarged compared to biopsy liver. This difference is most likely due to postmortem swelling of the peroxisomes. The peroxisomes have a flocculent empty-looking matrix (Fig. 1b) as though swelling and extraction of the contents has taken place. Although peroxisomes can readily be identified in autopsy tissue, their morphometric analysis can produce unreliable results due to postmortem swelling of the organelles with a subsequent increase in diameter.

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