Hepatic copper distribution in primary biliary cirrhosis shown by the scanning proton microprobe

DAVID JT VAUX,* F WATT,† GW GRIME,† J TAKACS,†

*From the *John Radcliffe Hospital, Oxford, and the †Department of Nuclear Physics, University of Oxford, Oxford*

SUMMARY A number of conditions are associated with abnormalities of trace metal handling by the liver. We report the application of the Oxford scanning proton microprobe to the analysis of hepatic copper in one such condition, primary biliary cirrhosis. The scanning proton microprobe analyses conventional tissue sections (5–10 μm thickness) and produces simultaneous elemental distribution maps of biologically relevant elements with a spatial resolution of 1 μm and a detection limit better than 1 ppm. We have confirmed the localisation of excess copper to periportal areas and suggest that such accumulation is confined to a proportion of periportal hepatocytes. We have also shown a close spatial correlation between regions of copper accumulation and areas of high sulphur concentration. The copper to sulphur ratio in these areas is consistent with their identity as aggregates of copper loaded metallothionein, and the scanning proton microprobe was further able to show that the aggregates contain <30 ppm zinc.

Primary biliary cirrhosis (PBC) is a chronic and progressively destructive non-suppurative cholangitis, which occurs mainly in women, usually over the age of 50. Small and medium intrahepatic bile ducts are surrounded and infiltrated by lymphocytes, plasma cells, eosinophils, and other less well defined cell types. Granulomas, which may be diffuse or well organised, are found near the damaged bile ducts. Later, the damaged bile ducts may be replaced by loose lymphoid aggregates and periportal fibrosis becomes prominent. Micronodular cirrhosis eventually supervenes, and in most cases death is due to end stage hepatic failure. The later stages of the condition are marked by greatly increased concentrations of copper in the liver¹ and other organs.² Abnormal copper concentrations can be shown by bulk analysis when large amounts of material are available.^{2,3} Unfortunately, averaged copper concentrations correlate poorly with clinical severity in PBC,⁴ and bulk analytic techniques cannot visualise the tissue distribution of elements. It is not possible from them to confirm or refute the hypothesis that accumulated intracellular copper is not hepatotoxic in PBC because it is rapidly sequestered within membrane bound organelles.⁵

Histological stains for copper lack specificity and sensitivity and depend on the physical state of the

metal.⁶ None the less, rubeanic acid and rhodamine staining have suggested that in PBC most accumulated copper is found in periportal hepatocytes (Fig. 1).

Material and methods

A standard Menghini needle liver biopsy specimen from a patient with PBC was divided into two parts; one part was immediately frozen in liquid nitrogen and the other was fixed and processed for routine histology, which included orcein and rubeanic acid staining. Frozen material from liver biopsies which were histologically normal was used as control frozen sections. This was processed in the same way as the PBC material.

Unstained frozen sections (7 μm thick) from the frozen part of each specimen were air dried on to 2 μm thick Mylar film in aluminium carriers and stored desiccated until examined using the Oxford scanning proton microprobe.

Proton induced x ray emission is a well validated technique for the measurement of elemental composition of many materials^{7,8}; it is based on the release of characteristic x rays from atoms excited by a high energy incident particle beam. Conventional electron beam microanalysis (particle energy ≈ 100 keV) has a detection limit of 50–100 ppm imposed by ineradicable x ray background (Bremsstrahlung),

Fig. 1 *Histological appearance of the liver in primary biliary cirrhosis. Orcein staining for sulphhydryl copper binding protein. Rubeanic acid staining for heavy metals, presumably copper, shows a similar distribution.*

a low x ray production rate, and problems of incident particle scattering even within ultrathin sections.⁹ With proton beam microanalysis (particle energy 2–4 MeV) Bremsstrahlung is greatly reduced, the detection limit for most elements of biological interest is 1–2 ppm, x ray production is considerably increased, and incident particle scatter is substantially reduced. The Oxford scanning proton microprobe utilises a rastered, focused proton beam to produce two dimensional elemental distribution maps with a spatial resolution of $\approx 1 \mu\text{m}$ from standard frozen histological sections or cell monolayers^{10,11} (for beam focusing to $1 \mu\text{m}$ see reference 12). This combination of the sensitivity and specificity of proton induced x ray emission with computer generation of two dimensional images means that the scanning proton microprobe can not only provide accurate measurements of elemental concentration on milligram quantities of biopsy material but can also show the distribution of these elements within the tissue.

For all examinations 4 MeV protons were used; the spectrums produced are reproduced as linear plots of x ray counts against x ray energy. Derived spectrums from small parts of the scanned area were produced by computer manipulation of the spatial distribution data. Full details of the design and construction of the microprobe have been published

elsewhere.^{11,12} The data acquisition and processing system have also been described.¹⁰

Results

Elemental spectrums from tissue sections of identically prepared biopsies from the livers of patients with histologically confirmed PBC (Fig. 1) and histologically normal livers are compared in Fig. 2a and b. Distorted, partially fibrosed, portal tracts were identified in stained frozen sections from the PBC biopsy and then located on the unstained section used for the scanning proton microprobe analysis. A $1 \text{ mm} \times 1 \text{ mm}$ scan over such an area generated the spectrum in Fig. 2a; the spectrum from a similar $1 \text{ mm} \times 1 \text{ mm}$ scan of normal liver is shown in Fig. 2b. The Table lists the elemental concentrations from these data and shows major differences, in particular a greatly increased concentration of copper in the PBC sample.

Fig. 3 shows the distribution of chlorine, sulphur, and copper in the sample: firstly, in the whole $1 \text{ mm} \times 1 \text{ mm}$ scan centred over a large portal tract (showing also several smaller portal tracts and the right hand edge of the biopsy) (Fig. 3a) and, secondly, at a higher magnification in a $200 \mu\text{m} \times 200 \mu\text{m}$ scan over the central top edge of this largest portal tract (Fig. 3b). Sulphur and chlorine are ubiquitous con-

Fig. 2 Energy spectra generated by the Oxford scanning proton microprobe. All spectra are linear plots of x ray counts against x ray energy. (a) 1 mm × 1 mm area from primary biliary cirrhosis liver (see text). Accumulated charge 7.26 μC. (b) 1 mm × 1 mm area from normal liver. Accumulated charge 10.0 μC. (c) Derived spectrum of 5 μm × 5 μm area centred on copper aggregate in primary biliary cirrhosis sample. (d) Derived spectrum of 5 μm × 5 μm area from area of cell adjacent to area of (c).

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Sample	Spectrum	Element							
		P	S	Cl	K	Ca	Fe	Cu	Zn
Normal	2 (b)	5420	1727	3301	695	1379	119	20	93
Primary biliary cirrhosis	2 (a)	4170	937	1673	1480	1117	49	130	20
Aggregate	2 (c)	2380	1402	1119	1247	439	30	2070	—
Surrounding cell	2 (d)	2630	450	735	781	703	40	93	—

The values are parts per million by weight, assuming that the liver sections are 7 μm thick and of density 1 g/cm^3 .

—Signifies below detection threshold.

stituents of biological material and their distribution clearly delineates the cellular areas of the section, showing a number of vessels and bile ducts. Potassium and phosphorus show similar distributions. In normal liver tissue copper concentrations are low and the distribution is homogenous (data not shown). Fig. 3 shows that the abnormal accumulation of copper in PBC is not homogenous; rather it occurs in small discrete areas with a diameter of 5–10 μm , which are found almost exclusively immediately around portal tracts. Fig. 3b shows that these accumulations of copper are not found at regular intervals around the periphery, which suggests that not every periportal hepatocyte is concerned with copper accumulation. The spatial correlation between copper and sulphur distributions apparent in Fig. 3b is confirmed by a number of scans over other copper rich regions. This association was further examined by generating elemental spectrums from 5 $\mu\text{m} \times 5 \mu\text{m}$ areas, firstly over a copper rich region (Fig. 2c) and then over an adjacent area of cell (Fig. 2d). The Table gives the average elemental concentrations from these areas: they show a 24-fold enrichment for copper and a more than threefold enrichment for sulphur within the copper and sulphur rich areas. The ratio of copper to sulphur measured from these spectrums is between 1.0 and 1.5 depending on the type of background subtraction employed (Table).

Conclusion

The abnormal hepatic copper of PBC has been measured by bulk analysis^{2,3} and some idea of its distribution has been obtained by light microscope histology⁶ and point analysis using electron beam based x ray techniques.⁴ The scanning proton microprobe offers the possibility, for the first time, of simultaneous determination of both quantity and distribution of copper in standard frozen sections of liver biopsy material.

The results in Fig. 2 confirm the quantitative abilities of the scanning proton microprobe and show a more than fivefold increase in average tissue copper concentrations in PBC. Examination of the

copper distribution in Fig. 3 shows that this is a dramatic underestimate of the increased copper concentrations in small areas within the periportal hepatocytes, where the concentration is at least 20 times greater than that of their surroundings and some hundred times the value measured in normal liver tissues.

The images in Fig. 3b suggest that the areas of high copper accumulation are not uniformly spaced around the portal tracts. This is consistent with the idea that not all periportal hepatocytes are concerned with the accumulation of excess copper in PBC. The association of copper with sulphur in the livers of patients with PBC was reported by Epstein *et al* using point analytic techniques.⁴ The scanning proton microprobe improves on point analysis by producing individual distribution maps for both copper and sulphur. We have shown that all of the sites of copper accumulation have high concentrations of sulphur. The computer system can also produce a distribution map of the ratio between copper and sulphur (not shown), and this confirms that the copper to sulphur ratio is roughly constant in all regions of copper accumulation, although the actual numerical value of this ratio depends on the background subtraction techniques employed.

The copper to sulphur ratio of between 1.0 and 1.5 is consistent with the identification of the copper rich areas as aggregates of metal loaded metallothionein. The metallothioneins are 6000 dalton metal binding proteins induced at the transcriptional level in hepatocytes by a heavy metal load (including copper, mercury, cadmium, and zinc).^{13,14} They contain about 30% cysteine residues and seven metal binding sites per molecule.¹⁵ Metallothionein isolated from normal adult human liver contains 6.7 g atoms/mol of zinc, 0.2 g atoms/mol cadmium, and 0.1 g atoms/mol copper,¹⁴ whereas metallothionein from neonatal human liver contains 2.4 g atoms/mol copper and only traces of other elements.¹⁶

It is clear from Fig. 2c that the copper and sulphur rich aggregates are deficient in zinc, the K_{α} peak for which lies between the copper K_{α} and K_{β} peaks. The zinc concentration in these areas is below 30 ppm. If the aggregates represent metallothionein loaded

Fig. 3 Elemental distribution maps from the primary biliary cirrhosis liver biopsy. (a) 1 mm $\times$ 1 mm area over portal tract (see text). (b) 200 μ m $\times$ 200 μ m area scan from the upper right border of the portal tract seen in (a). Concentrations are encoded in colours based on a division of the range from zero to tissue maximum into 16 sectors. The colour code used is displayed beneath each elemental map. Because the maximum value is element specific, direct comparison between elements is not possible with this visual scaling.

with copper and correspond to the membrane bound organelles described on electron microscopy by Epstein *et al*⁴ then it is possible that the lack of apparent copper hepatotoxicity in PBC is due to rapid copper sequestration in a zinc deficient particulate metallothionein aggregate. Riordan and Richards have found such a copper rich, zinc deficient metallothionein in the particulate fraction of human fetal liver,¹⁷ where it may explain the lack of copper hepatotoxicity despite normally high concentrations of the element.

The scanning proton microprobe should prove a powerful new tool in the analysis of the biological role of specific elements, including heavy metals.

We thank Professor JO'D McGee, Department of Pathology, John Radcliffe Hospital, Oxford, for providing the samples and for invaluable discussion throughout this work.

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Requests for reprints to: Dr David JT Vaux, John Radcliffe Hospital, Headington, Oxford, England