A Biochemical Comparison of Normal Human Liver and Hepatocellular Carcinoma Ferritins

Sarah BULLOCK, Adrian BOMFORD and Roger WILLIAMS
Liver Unit, King's College Hospital and Medical School, Denmark Hill, London SE5 9RS, U.K.

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1. The iron contents, gel migration rates and isoelectric-focusing patterns of normal liver and hepatocellular carcinoma ferritins from the same patients were compared. 2. Sucrose-density-gradient centrifugation showed that the number of iron atoms per ferritin molecule was decreased to approximately half in carcinoma tissue when compared with normal liver. 3. On electrophoresis, hepatocellular carcinoma ferritin migrates faster and is therefore more negatively charged than normal liver ferritin, thus refuting the general view that the more negatively charged a ferritin molecule the greater its iron content. 4. Comparison of tumour and normal liver ferritin subunit compositions on acid/urea/polyacrylamide gels showed hepatocellular carcinoma ferritin to contain an additional, more negatively charged, subunit to normal liver ferritin. 5. Isoelectric focusing showed that hepatocellular carcinoma tissue contains isoferitins with isoelectric points intermediate between the ranges of normal liver and normal heart isoferitins.

It is now generally accepted that the iron-storage protein ferritin comprises multiple stable molecular forms or isoferitins that are unchanged by alterations in pH (Alpert et al., 1973; Granick, 1946). These isoferitins have been separated by isoelectric focusing or ion-exchange chromatography (Drysdale, 1974; Urushizaki et al., 1973). High tissue concentrations of ferritin are known to occur in a number of neoplastic disorders, including leukaemias, lymphomata and solid tumours (Jacobs & Worwood, 1975). As well as the isoferitins found in normal cells, abnormal ferritins have been isolated from malignant cell lines (Richter & Lee, 1970), rat and human hepatocellular carcinoma (Urushizaki et al., 1973; Alpert et al., 1973) and pancreatic carcinoma (Marcus & Zinberg, 1974). Richter (1965) and Richter & Lee (1970) found that ferritin isolated from malignant cells in tissue culture and from rat hepatoma migrated faster than normal towards the anode during electrophoresis. Alpert et al. (1973) confirmed the existence of specific acidic isoferitins in human liver carcinoma, and also showed the presence of these variants in foetal liver tissue. These tumour isoferitins were subsequently identified in foetal tissue obtained up to 10 weeks' gestation (Alpert, 1975) and have been designated 'carcinofoetal isoferitins'.

On the basis of previous studies (Bomford et al., 1978) we have suggested the multiple isoferitins of normal human liver consist of two subunits that are virtually identical in molecular weight but differ in net charge, whereas human liver carcinoma ferritin comprises the same two subunits as well as a third, unique, subunit (Alpert, 1975). It is thought that differing proportions of these subunits may account for the several normal isoferitins and unique tumour-specific acidic isoferitins. Evidence from experiments performed in vitro, however, suggests that isoferitins also exhibit a functional heterogeneity with respect to iron content in relation to their ability to take up iron (Harrison et al., 1977). In our own studies we were able to demonstrate a correlation between the iron content of human tissue isoferitins and their structure in terms of isoelectric point and subunit composition (Bomford et al., 1978).

In an attempt to resolve some of these differences, we have studied the iron content and distribution of isoferitins in samples of normal liver and hepatocellular carcinoma tissue. Wherever possible, normal and malignant liver tissue from the same patients has been used in order to achieve more valid comparisons and also to compensate for the wide variation in ferritin content of the control livers (Crichton et al., 1973).

Materials and Methods

Chemicals

Analytical-reagent-grade biochemical reagents were obtained from Hopkin and Williams, Romford, Essex, U.K. Polyacrylamide and NN'-methyl-
enebisacrylamide were from Eastman Organic Chemicals, Rochester, NY, U.S.A. Ammonium persulphate and NNN'N'-tetramethyl-1,2-diaminomethane were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Carrier ampholytes (pH 3.5–10.0) were from LKB Produkter AB, Bromma, Sweden.

Human tissue

Fresh human tissue samples were obtained at post-mortem between 8 and 10h after death. The bodies were refrigerated at 4°C before post-mortem and after removal the tissues were stored at −25°C until use. Twelve livers were used from three females and nine males between the ages of 25 and 63 who had been investigated at the Liver Unit, King’s College Hospital, and treated for primary hepatocellular carcinoma. All the tissue used was from patients who did not have cirrhosis. Separation of normal liver and hepatocellular carcinoma was based on histological appearance under a light-microscope at ×50 magnification together with macroscopic investigation.

Ferritin preparation

Ferritin was prepared by the method of Drysdale & Munro (1965) with the omission of the ion-exchange chromatography step. A third step was added comprising gel-filtration chromatography (gel bed 90 cm × 2.5 cm) on Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden) with phosphate-buffered saline, pH 7.2 (120 mM-NaCl in 15 mM-sodium phosphate buffer), as the eluting buffer. This separated monomeric ferritin from oligomeric forms of the protein (Niitsu & Listowsky, 1973) and, after concentration by ultrafiltration with M50 membranes (Amicon Corp., Lexington, MA, U.S.A.), monomeric ferritin was used in our studies. The purity of each preparation was assessed by Laurell (1965) ‘rocket’ immunoelectrophoresis with a monoclonal rabbit anti-(normal liver ferritin) or anti-(hepatoma ferritin) serum. Polyacrylamide-gel electrophoresis normally showed only bands that stained for both protein and iron and that occurred in positions where ferritin is known to migrate. Some of the preparations, however, contained small traces of albumin, seen as a band with high anodal mobility and strong affinity for the Bromophenol Blue tracking dye. As all our determinations of ferritin were carried out with monospecific antisera this was not considered to be a problem. Ferritin solutions at concentrations between 500 μg/ml and 2.5 μg/ml were stored at 4°C in deionized water containing 0.05% NaN₃.

Protein determinations were carried out by the method of Lowry et al. (1951), with fat-free human serum albumin as a standard. Ferritin iron and total liver iron were measured by atomic absorption spectrophotometry with a Perkin–Elmer instrument with a heated graphite furnace and automatic sample loader. Ferritin solutions appropriately diluted were injected into the furnace without previous treatment.

Preparation of antisera

Antiserum to normal human liver ferritin and to hepatocellular carcinoma ferritin was prepared by injection of purified preparations of these proteins. A 1:1 mixture of ferritin solution and Freund’s incomplete adjuvant containing approx 1.0 mg of ferritin protein, as determined by the Lowry et al. (1951) method, was injected intravenously into each rabbit. Subsequent booster injections of ferritin alone were administered subcutaneously at weekly intervals. The antisera obtained gave a single precipitin line on agar-gel diffusion when tested against purified human liver ferritin or hepatocellular carcinoma ferritin as appropriate. After absorption of the antiserum by excess purified human liver ferritin or hepatocellular carcinoma ferritin, no precipitin line was detected with normal human serum that had been absorbed into an excess of antiferritin serum.

Density-gradient centrifugation

Discontinuous gradients were layered with 2 ml each of 25%, 20%, 15%, 10% and 5% (w/v) sucrose in phosphate-buffered saline, pH 7.2 (120 mM-NaCl in 15 mM-sodium phosphate buffer). Then 1–2 mg samples of ferritin in 1 ml of phosphate-buffered saline, pH 7.2, were centrifuged on these gradients in a 3 × 23 ml swing-out rotor on an MSE Superspeed 65 centrifuge at 79 500 g for 100 min at 4°C. Ten 1 ml fractions were collected from the top of the gradients with a syringe and fine needle. Initial experiments proved this to be both the easiest method to use for the removal of fractions and the one that caused least disturbance of the gradient. The concentration of ferritin protein in each fraction was measured by using the Laurell (1965) ‘rocket’ technique. A monoclonal rabbit antiserum to purified human liver ferritin or to hepatoma ferritin was used as appropriate. Standards were purified human liver or hepatoma ferritin in which the protein concentration was measured by the method of Lowry et al. (1951). Ferritin iron in each fraction was measured by atomic absorption spectrophotometry.

Polyacrylamide-gel electrophoresis

Gels (3 mm × 130 mm × 180 mm) containing 4.8% (w/v) acrylamide and 0.2% (w/v) methylenebisacrylamide were cast and run in a vertical slab-gel apparatus (Shandon Southern Instruments, Camberley, Surrey, U.K.). Gel buffer was 76 mM-Tris/citrate, pH 8.9, and electrode buffer was 82 mM-Tris/borate, pH 8.3, containing 3.4 mM-EDTA (disodium salt). Bromophenol Blue was used as a track-
ing dye. Samples were electrophoresed at 10°C in accordance with the manufacturer’s instructions. Polyacrylamide-gel electrophoresis of ferritin subunits was carried out in gels containing 6.25 M-urea, pH 3.2, by the method of Panym & Chalkley (1969). Samples were run at 10°C in gel rods (7 mm x 8 mm) containing 15% (w/v) acrylamide and 0.1% (w/v) methylenebisacrylamide in a Shandon disc electrophoresis apparatus at 3 mA/gel for 3–4 h. Methyl Green was used as the tracking dye.

**Gel electrofocusing**

This was carried out in flat beds (1.5 mm x 120 mm x 250 mm) containing 4% (w/v) acrylamide, 0.16% (w/v) methylenebisacrylamide and 2% (w/v) carrier ampholytes (pH 3.5–10.0) in an LKB Multiphor apparatus. Samples were run at 4°C in accordance with the manufacturer’s instructions. The pH gradient in these gel electrofocusing experiments was determined as described by Vesterberg (1972).

Ferritin was dissociated in 67% (w/v) acetic acid at 0°C for 1 h as described by Harrison & Gregory (1968), and the resulting subunits were dialysed overnight against 0.9 M-acetic acid/6 M-urea. This mixture was then applied directly to denaturing gels (containing 0.9 M-acetic acid).

Gels were stained for protein with 0.025% (w/v) Coomassie Blue R in methanol/acetic acid/water (3:1:8, by vol.) for 12 h at 19°C and cleared in repeated changes of the solvent.

**Results**

**Iron content of normal liver and hepatoma ferritin**

Since distinction between normal and malignant liver tissue was based on histological evaluation of small samples and macroscopic examination, the possibility cannot be excluded that small amounts of tumour were contained within apparently normal areas of tissue. The results obtained for these normal specimens, however, correlated well with those for liver tissue from healthy adults, and so visual differentiation of normal and hepatoma tissue was regarded as adequate for the purposes of this study. Table 1 shows the ferritin protein contents, measured by Laurell (1972) ‘rocket’ immunoelectrophoresis and ferritin iron contents, measured spectrophotometrically, of the samples studied. Although the range in concentration is wide, as is found in normal populations, the tissue concentration of ferritin was decreased in hepatocellular carcinoma. The concentration of iron also decreases to a much greater extent than the ferritin protein, thus leading to a significant decrease in the number of iron atoms per ferritin molecule in the carcinoma state.

These findings were confirmed by sucrose-density-gradient centrifugation of the ferritin preparations, in which ferritin types were separated according to their molecular iron content. A clear difference in distribution profiles emerges between normal liver and hepatocellular carcinoma tissue from the same individuals (Fig. 1). These patterns were consistent throughout all our samples. Hepatocellular carcinoma tissue always contained ferritin with a much higher proportion of the molecules distributing near the top of the gradient, and thus having a lower iron content than normal liver and hepatoma liver ferritin.

**Electrophoretic mobility of normal liver and hepatoma ferritin**

Examination of the electrophoretic mobilities of the various ferritin preparations in 5%-(w/v)-polyacrylamide gels gave unexpected results. In both stick gels and large slab gels (where all the samples were applied to the same gel), the tumour ferritin preparations migrated slightly but reproducibly faster towards the anode and thus were more nega-

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Table 1. Ferritin protein and ferritin iron concentrations in crude homogenates of normal livers and hepatomas

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Type of tissue</th>
<th>Ferritin protein (μg/g of tissue concn.)</th>
<th>Ferritin iron (μg/g of tissue concn.)</th>
<th>Iron content (μg/g of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal liver</td>
<td>1286</td>
<td>187</td>
<td>0.145</td>
</tr>
<tr>
<td>1</td>
<td>Hepatoma</td>
<td>885</td>
<td>79</td>
<td>0.089</td>
</tr>
<tr>
<td>2</td>
<td>Normal liver</td>
<td>3405</td>
<td>409</td>
<td>0.120</td>
</tr>
<tr>
<td>2</td>
<td>Hepatoma</td>
<td>1841</td>
<td>36</td>
<td>0.019</td>
</tr>
<tr>
<td>3</td>
<td>Normal liver</td>
<td>344</td>
<td>121</td>
<td>0.353</td>
</tr>
<tr>
<td>3</td>
<td>Hepatoma</td>
<td>386</td>
<td>58</td>
<td>0.150</td>
</tr>
<tr>
<td>4</td>
<td>Hepatoma</td>
<td>365</td>
<td>48</td>
<td>0.131</td>
</tr>
<tr>
<td>5</td>
<td>Hepatoma</td>
<td>531</td>
<td>54</td>
<td>0.101</td>
</tr>
<tr>
<td>6</td>
<td>Control normal</td>
<td>632</td>
<td>108</td>
<td>0.170</td>
</tr>
<tr>
<td>7</td>
<td>Control normal</td>
<td>618</td>
<td>139</td>
<td>0.220</td>
</tr>
<tr>
<td>8</td>
<td>Control normal</td>
<td>723</td>
<td>135</td>
<td>0.190</td>
</tr>
</tbody>
</table>
tively charged than the normal ferritins, as shown in Fig. 2. These differences could not be attributed to differences in sample loading since the same amount (10–15 μg) of protein was applied in each case.

Subunit composition of normal liver and hepatoma ferritin

In order to compare the subunit composition of hepatocellular carcinoma ferritin with that of normal liver ferritin, the samples were treated with 6M-urea in 96% (w/v) acetic acid to break down the inter-subunit linkages, and were then electrophoresed on polyacrylamide gels. The gels themselves contained acetic acid and urea in order to prevent reassociation of the subunits during electrophoresis. Typical gels are shown in Fig. 3. It can be seen that the carcinoma ferritin contained an additional, more negatively charged, subunit to the normal liver ferritin, and this seemed to correspond to the more basic of the two heart ferritin subunits (Linder et al., 1975; Bomford et al., 1977). The additional, faster-running, band seen on the gel containing hepatoma ferritin is due to contamination of the sample with albumin. This was shown by elution of this band.
from the gel and subsequent testing of the recovered material by immunodiffusion against anti-albumin and anti-(hepatoma ferritin) sera. There was a strong precipitin reaction with anti-albumin but none with anti-(hepatoma ferritin) sera.

**Separation of isoferritins**

The possibility that the apparent similarity between one of the heart ferritin and one of the hepatocellular carcinoma ferritin subunits is chance was borne out by the results of isoelectric-focusing experiments. Typical results obtained after focusing various ferritin preparations in a polyacrylamide gel slab are shown in Fig. 4. The patterns show that hepatocellular carcinoma isoferritins occur at different points in the gradient from those found in heart tissue and have isoelectric points that are intermediate between those of the normal liver and normal heart isoferritin ranges.

**Discussion**

These results have shown that the isoferritins associated with hepatocellular carcinoma are more acidic than those occurring in normal hepatocytes. Similar acidic isoferritins have been shown to occur in human pancreatic and breast tumours (Marcus & Zinberg, 1974) and in HeLa cells (Drysdale & Singer, 1974). Alpert (1975) showed that acidic tumour ferritin has a molecular weight identical with that of the more basic normal liver ferritin.

In other experiments we have found that the acidic hepatoma isoferritins are present in foetal liver in early gestation, disappear in the second trimester and reappear in cancer tissue. This agrees with the findings made by Alpert et al. (1973). Thus these isoferritins are another example of carcinofetal alteration of protein synthesis in tumours.

In the present material the tissue concentration of ferritin was decreased in hepatocellular carcinoma and the amounts of iron contained by the ferritin were much lower in hepatocellular carcinoma than in normal liver. Niitsu et al. (1975) and Reissman & Dietrich (1956) have published similar results. Thus malignancy is associated with a shift towards a predominance of less-heavily iron-loaded ferritins than in normal liver. Bearing in mind that the function of ferritin in tissues is to store iron, this suggests that hepatocellular carcinoma and liver ferritins differ both structurally and functionally. However, Niitsu et al. (1975) could not distinguish immunologically
between hepatocellular carcinoma and normal liver ferritin, although some quantitative differences in antibody affinity have been shown (Linder et al., 1975).

In earlier studies we have found (Bomford et al., 1978) and others have also reported (Wagstaff et al., 1978) that the isoelectric points of isoferritins decrease as their iron contents increase, so that the most dense sucrose-density-gradient fractions contain the most acidic isoferritins. Thus our initial observation of the very low iron content of ferritin in liver cancer suggested the production of more basic isoferritins than in the normal liver spectrum for the same individual. There is also a possibility that preferential breakdown of the more acidic components occurs. However, electrophoresis and isoelectric-focusing experiments showed that the correlation between increasing iron content and decreasing isoelectric point that is found in normal human tissues, including liver and heart, does not apply to hepatocellular carcinoma tissue. This contradiction lends weight to the theory that the surface charge of isoferritins is not influenced by their iron content, which is situated within the protein shell (Farrant, 1954), but is due to their subunit composition (Arioso et al., 1976; Bomford et al., 1977; Drysdale, 1977). It does not agree with the hypothesis that increasing amounts of the more acidic subunits in isoferritins lead to higher iron-binding capacities (Wagstaff et al., 1978). However, the present studies confirm our previous findings (Bomford et al., 1977) that molecules containing a higher proportion of acidic subunit could be isolated from heart tissues with a wide range of iron loading. Thus it seems that some factor other than subunit composition is the principal determinant of the amount of iron taken up by a particular isoferritin molecule.

Despite clear differences between hepatocellular carcinoma and normal liver ferritins, the presence of more acidic isoferritins in normal tissues such as heart and kidney has complicated the identification of carcinofetal ferritin. Marcus & Zinberg (1975) and Drysdale et al. (1975) suggested that the presence of carcinofetal isoferritins may reflect a phenotypic shift in cancer in the distribution of tissue-specific isoferritins from liver type to heart type in the same manner as for lactate dehydrogenase (Weinhouse et al., 1972). However, the fact that the iron content of hepatocellular carcinoma ferritin is so small relative to that of normal acidic tissue isoferritins suggests that this may not be the case. Further structural analysis is needed to elucidate the relationship between the normal tissue and tumour isoferritins.

Increased production of ferritin by tumour cells has been shown in acute leukaemia (White et al., 1974; Mori et al., 1975), but we found low concentrations of ferritin in hepatocellular carcinoma in comparison with normal liver. However, significant elevations of serum ferritin concentrations have been found in patients with liver tumour (Buffe et al., 1970; Alpert et al., 1973; Niitsu et al., 1975). Thus it is possible that hepatocellular carcinoma tissue produces a particular type of ferritin that is secreted into the circulation rather than accumulated in the tissue. Indeed, Niitsu et al. (1975) have shown that some of the serum ferritin present in patients with malignant disease is derived from the tumour tissue. Secreted proteins are normally synthesized mainly on membrane-bound polyribosomes (Puro & Richter, 1971; Konijn et al., 1973). It has been shown that even in normal rat liver some of the ferritin is synthesized on polyribosomes attached to the endoplasmic reticulum (Konijn et al., 1973).

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References

Konijn, A. M., Baliga, B. S. & Munro, H. N. (1973) FEBS Lett. 37, 249–252
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