Serum and tissue α-L-fucosidase activity in the pre-clinical and clinical stages of hepatocellular carcinoma

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SUMMARY
1. To assess the value of serum α-L-fucosidase (EC 3.2.1.51) as a marker for hepatocellular carcinoma, the activity was measured in patients with hepatocellular carcinoma and in control subjects.
2. Mean serum α-L-fucosidase activity was significantly greater in 35 patients with hepatocellular carcinoma (225 ± 69 nkat/l) than in 35 patients with cirrhosis and 20 normal subjects (134 ± 30 and 93 ± 28 nkat/l, respectively). The overlap between hepatocellular carcinoma and cirrhosis, however, was such as to severely limit any value of the enzyme as a diagnostic test.
3. In four cirrhotic patients with hepatocellular carcinoma, an increased enzyme activity was detectable in samples taken up to 66 months before the tumours were diagnosed clinically.
4. The serum activity of α-L-fucosidase fell to within the reference range after liver transplantation for hepatocellular carcinoma in three patients and in one of these a subsequent rise was associated with tumour recurrence which was diagnosed at 8 months after the rise in activity. Ineffective cytotoxic chemotherapy was also associated with a progressive rise in serum α-L-fucosidase activity.
5. α-L-fucosidase activity in tumour tissue was significantly lower than that seen in non-tumour tissue from cirrhotic patients. These reductions may represent increased transport from the tissue and may partly account for the increased serum activity found in some patients with hepatocellular carcinoma.

Key words: cirrhosis, α-L-fucosidase, hepatocellular carcinoma.

Abbreviations: AFP, α-fetoprotein; CAH, chronic active hepatitis; HCC, hepatocellular carcinoma; PBC, primary biliary cirrhosis.

INTRODUCTION
α-L-fucosidase (EC 3.2.1.51) is a lysosomal enzyme involved in the catabolism of fucose-containing glycoproteins in several tissues including liver. The activity of this and other lysosomal enzymes has been reported to be altered in several Morris hepatoma cell lines and in a number of malignant diseases [1-4], where it is thought to represent disturbances in carbohydrate metabolism [5, 6]. Increased serum levels have also been reported among patients with hepatocellular carcinoma (HCC) leading to the suggestion that this enzyme may act as a useful marker for HCC development [7, 8].

In this communication we report investigations on the serum and tissue activity of α-L-fucosidase in patients with HCC and cirrhosis. In addition, α-L-fucosidase was measured in serial serum samples from cirrhotic patients, some of whom developed HCC, and in others undergoing therapy with cytotoxic agents or orthotopic liver transplantation.

MATERIALS AND METHODS
Subjects and protocol
Serum α-L-fucosidase activity was measured in 35 patients with HCC (27 males, eight females) aged 20–73 years. The diagnosis was established in each case by histological examination of tumour tissue. Twenty-eight had associated cirrhosis [alcoholic cirrhosis, eight; cryptogenic cirrhosis, five; primary biliary cirrhosis (PBC) one; chronic active hepatitis (CAH), 12; haemochromatosis, two]. The two control groups comprised 35 patients with uncomplicated cirrhosis [alcoholic cirrhosis, nine; cryptogenic cirrhosis, eight; PBC, eight; CAH, 10] and 20 healthy volunteers recruited from the hospital staff.

Serial sera from seven patients with histologically confirmed cirrhosis (CAH, four; alcoholic cirrhosis, one; PBC, two), none of whom displayed any clinical or radiological evidence of HCC at the time of the first available sample, were identified from the Unit's serum bank. In
judged by ultrasound examination and serum a-feto-

Fig. 1. Serum α-1-fucosidase activity (○) and serum AFP level (○) in the various subject groups. The upper limit of the normal range (mean ± 2 sds) for serum α-1-fucosidase activity is indicated (— — —).

each case at least three, and up to seven, samples were available over a period of between 2 and 6 years. Four of these patients had been chosen because of the ultimate development of HCC, the diagnosis of which was established at or around the time of obtaining the final sample. Selection of the three patients who did not develop HCC was based on the availability of samples covering similar time periods to those over which the patients developing HCC were studied, and no assessment of α-1-fucosidase in either group had been made previously.

The effect of cytotoxic chemotherapy was studied in three patients with HCC (two females, one male; one had CAH and two were non-cirrhotic). Of these, two received four courses of intra-arterial mitoxantrone (7.5 mg/24 h) over 3 consecutive days and the third was given five courses of intravenous doxorubicin (60 mg/m²) at 3–4 week intervals. Two patients had progressive disease as judged by ultrasound examination and serum α-fetoprotein (AFP) levels, and one had stable disease over the period of study.

Serum samples were also obtained from three patients with HCC (one female, two males) before orthotopic liver transplantation with additional samples being collected at intervals after transplantation for periods of up to 12 months. All three patients had elevated serum AFP levels before operation. One of these patients died at 7 months from recurrent hepatitis B infection, the second developed histologically proven tumour recurrence at 10 months, while the third remained alive and well with no evidence of tumour reappearance at 10 months.

Preparation of tissue cytosols

For the preparation of cytosols, liver tissue was obtained from nine patients (five males, four females), aged 22–44 years, undergoing liver transplantation. Of these, five had HCC (three males, two females) and in two cases this was accompanied by underlying cirrhosis. The remaining four patients had histologically confirmed cirrhosis without HCC (CAH). The samples were stored at −70°C immediately after hepatectomy and were not thawed until just before cytosol preparation. On the basis of macroscopic and microscopic examination, it was possible to distinguish between tumour and non-tumourous tissue of the same liver from four patients with HCC, and separate cytosols were prepared in such cases.

Cytosols were prepared by methods used previously for obtaining extracts of rat liver [9] and tadpole liver [10]. Tissues were finely minced at 4°C in 10 mmol/l sodium phosphate buffer, pH 7.4, containing 150 mmol/l NaCl. Small portions were homogenized for 15–20 s with an Ultraturrax homogenizer (Janke-Kunkel, Germany). Samples were then subjected to sonic disintegration at 4°C by an MSE Soniprep 150 (MSE, Crawley, West Sussex, U.K.) using three bursts of 10 s duration at 24 kycles/s at an amplitude of 10 u. The resulting preparations were centrifuged at 106 000 g for 1 h. The supernatants obtained were used for estimations of α-1-fucosidase, α-D-mannosidase (EC 3.2.1.24) and α-glucosidase (EC 3.2.1.20) activities and of total protein.

In order to check the efficiency of enzyme extraction from tumour and non-tumour tissue, the pellet remaining after removal of the supernatant from the above procedure was, in four cases (two patients with HCC, two cirrhotic patients), subjected to two further extractions, by which any co-sedimented α-1-fucosidase could be estimated. This involved washing the pellets in the extraction buffer, followed by homogenization and sonic disintegration as described above. The final pellet was resuspended in 50 mmol/l Tris buffer, pH 7.0. The activity of α-1-fucosidase in all washings, supernatant layers and resuspended pellet was determined.

Estimations of glycosidase activities and AFP levels

Sera and tissue cytosols were stored at −20°C before analysis. α-1-Fucosidase in serum and cytosol was measured with p-nitrophenyl-α-fucoside artificial substrate by the method of Van Hoof & Hers [11]. The acidic (lysosomal) forms of α-D-mannosidase and α-glucosidase were measured at pH 4.0 using appropriate p-nitrophenyl substrates [12–14]. Serum α-1-fucosidase activity is

Table 1. Serum α-1-fucosidase activity in cirrhotic patients

<table>
<thead>
<tr>
<th>Type of cirrhosis</th>
<th>α-1-Fucosidase activity (nkat/l)</th>
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<tbody>
<tr>
<td>Alcoholic cirrhosis (n = 9)</td>
<td>160 ± 73</td>
</tr>
<tr>
<td>Cryptogenic cirrhosis (n = 8)</td>
<td>101 ± 48</td>
</tr>
<tr>
<td>PBC (n = 8)</td>
<td>147 ± 57</td>
</tr>
<tr>
<td>Autoimmune CAH (n = 10)</td>
<td>127 ± 54</td>
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Values are means ± SD.
Serum and tissue α-L-fucosidase in hepatocellular cancer

Fig. 2. Serial measurements of serum α-L-fucosidase activity (a) and serum AFP level (b) in patients with cirrhosis. Patients did (●) or did not (○) develop HCC, which was diagnosed at the points shown (O). The upper limits of the normal ranges (mean ± 2 sds) are indicated (---). The cirrhotic group was observed among those patients with alcoholic cirrhosis (Table 1).

Serial measurements in the four cirrhotic patients who ultimately developed HCC revealed fluctuations in serum α-L-fucosidase activity at levels consistently above the established reference range from the time of the first sample in each case (Fig. 2a). In contrast to these findings, those cirrhotic patients not developing HCC displayed values which remained either within or below the normal range throughout the period of measurement (Fig. 2a).

In the two patients treated with mitoxantrone, serum α-L-fucosidase activity remained fairly constant, but above the normal range, during a 12 month follow-up period in the patient with stable disease and displayed a steady rise over a 4 month period in the patient with progressive disease (Fig. 3a). In the single patient receiving doxorubicin, serum α-L-fucosidase activity remained at the upper limit of the normal range during a 3 month period. The three patients who underwent liver transplantation showed a fall in serum α-L-fucosidase activity of between 18 and 53% during the 2 months immediately after operation to within the normal range (Fig. 4a). All three patients subsequently displayed increasing serum α-L-fucosidase activity, but in only one of the three was expressed as nkat/l, and tissue enzyme activities are expressed as nkat/mg of protein. The serum AFP level was determined by a commercially available enzyme immunoassay method (Abbott Diagnostics, Maidenhead, Berks, U.K.) and is expressed in ng/ml. Protein was estimated with the bicinchoninic acid protein assay reagent (Pierce, Luton, Beds, U.K.) by the method of Smith et al. [15]. In all cases, enzyme activities are presented as means ± sd, and differences between the various groups were assessed by using the Mann–Whitney U-test. Wilcoxon's test for paired variables was used when comparing activities in tumour and non-tumour tissue from the same patients with HCC.

RESULTS

The mean serum α-L-fucosidase activity was significantly elevated in patients with HCC (225 ± 69 nkat/l, mean ± sd) compared with cirrhotic patients (134 ± 30 nkat/l, P < 0.05) and normal subjects (93 ± 28 nkat/l, P < 0.01) (Fig. 1). In the HCC group, 63% of patients displayed values above the mean plus 2 sds of the normal control group value, whereas among cirrhotic patients, 43% exceeded this value. The highest activity within the cirrhotic group was observed among those patients with alcoholic cirrhosis (Table 1).

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elevated in two patients receiving mitoxantrone and doxorubicin and was consistently within the normal range in the remaining mitoxantrone-treated subject (Fig. 3b). In those who underwent orthotopic liver transplantation, there were marked reductions to levels within the normal range in the 2 months immediately after the operation (Fig. 4b). These reductions preceded those in serum $\alpha$-fucosidase activity by between 2 and 6 weeks.

**Tissue cytosol glycosidase activities**

Activities of $\alpha$-fucosidase in tumour tissue from patients with HCC were significantly lower than those in non-tumour tissue from both patients with HCC and cirrhotic patients ($P<0.05$, Table 2). Among HCC patients, however, activities in tumour and non-tumour tissue cytosols did not differ significantly, although the former were either lower (two cases) or remained unchanged (Table 2). There were no significant differences between the activities of acidic $\alpha$-D-mannosidase or $\alpha$-glucosidase measured in tumour and non-tumour tissue (Table 2). That the reduced $\alpha$-fucosidase activity in tumour tissue cytosol accurately reflects the pattern of change in affected tissue was indicated by comparison of activities remaining in pelleted material after initial ultracentrifugation of homogenates and removal of the supernatants. In all cases, total activity remaining bound to the pellet and subsequently released by further extractions amounted to less than 2% of that determined in cytosolic extracts.

**DISCUSSION**

Although mean serum $\alpha$-fucosidase activities were significantly greater in patients with HCC than among the two control groups, there was considerable overlap between the individual values in the HCC and cirrhotic groups. Such findings are at variance with the observations of Deugnier et al. [8], who reported minimal overlap between patients with HCC and those with cirrhosis. The presence of tumour was found, by these authors, to be accompanied by serum activities of $\alpha$-fucosidase in excess of 110 nkat/l in 75% of cases, unlike the situation in the cirrhotic patients, where at least 90% had values below this level.

A possible explanation for the discrepancies might lie in different aetiologies of the groups investigated. Among 36 cirrhotic control subjects studied by Deugnier et al. [8], 35 had alcoholic cirrhosis, whereas in our study the cirrhotic group comprised roughly equal numbers of patients with alcoholic cirrhosis, CAH, cryptogenic cirrhosis and PBC. Although it is apparent that there are differences in cirrhosis according to aetiology, marked overlap persists between the cirrhotic and HCC patients whichever type of cirrhosis is considered. The patients with alcoholic cirrhosis displayed the highest serum activities of $\alpha$-fucosidase and these values are greatly in excess of those reported by Deugnier et al. [8]. It is also unlikely that differences in methodology explain the discrepancies between $\alpha$-fucosidase activities observed.

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**Fig. 4.** Serum $\alpha$-fucosidase activity (a) and serum AFP level (b) in three patients with HCC after orthotopic liver transplantation. Samples were obtained at various times after transplantation, which was carried out at time 0. Tumour recurrence is indicated (O).

Mean values of serum AFP levels in the same groups of subjects showed less overlap between patients with HCC and those with non-malignant disease. The serum AFP levels in 35 patients with HCC ranged from 0 to $3 \times 10^5$ ng/ml (median 600 ng/ml), whereas 32 of the 35 cirrhotic patients had levels of between 0 and 50 ng/ml (Fig. 1). Of the four cirrhotic patients who developed HCC and in whom serum $\alpha$-fucosidase activity was measured serially, the serum AFP level exceeded the normal range at time points 6 to 21 months before the diagnosis of HCC in three cases. Diagnostic levels (>500 ng/ml) were exceeded in two of these at 5 and 9 months before confirmation of the disease (Fig. 2b). For the fourth patient and those patients who did not develop HCC, serum AFP levels remained below 50 ng/ml throughout the period of observation. Among the patients with HCC treated by cytotoxic chemotherapy, serum AFP levels remained...
for individual groups in the present studies and those carried out by Deugnier et al. [8]. Sera were similarly stored (−20°C) and the enzyme was assayed in both instances by the use of p-nitrophenyl-α-L-fucopyranoside as artificial substrate at pH 5.0–5.5. Indeed, a similar method was also used by Bukofzer et al. [16], who reported much higher mean activities in patients with HCC than those determined in the present study or in other liver-derived glycosidases. In common with other α-L-fucosidase activity, which the two major components display PI values of 4.68 and 4.84 while the minor forms occur between PI values of 4.52 and 4.96 [19, 20]. In both cases sialic acid plays a major role in conferring acidity on the more anodal forms of the protein [18, 19]. This carbohydrate has a strong influence on the clearance rates of glycoproteins from the circulation, generally increasing the time required for uptake by peripheral tissues [21, 22]. The more acidic nature of α-L-fucosidase may be due to greater sialic acid content, which would increase the time required for removal of elevated quantities from circulation after transplantation.

In view of the increased activity of α-L-fucosidase in serum from HCC patients, the decrease observed in cytosols prepared from tumour tissue were somewhat surprising but were in agreement with the findings of Leray et al. [23]. On this basis, it is tempting to speculate that the serum elevations in HCC may, at least in part, be caused by increased transport from tumour tissue. This is particularly so in view of the failure to detect any α-L-fucosidase activity in pelleted material despite repeated extractions. Although we found no overall significant difference between activities in cytosols prepared from tumour and non-tumour tissue taken from the same HCC patient, our numbers were small and in three of the four cases the activity in tumour tissue was less than that in non-tumour tissue, again in close agreement with the findings of Leray et al. [23].

It seems likely, moreover, that the effects of the presence of tumour on enzyme activity do not extend to other liver-derived glycosidases. In common with other hydrolyses examined previously [24], the activities of α-L-mannosidase and α-glucosidase did not differ significantly in tumour and non-tumour tissue, suggesting that the observed changes are specific to α-L-fucosidase.

Alterations in α-L-fucosidase activity affect fucose turnover within hepatocytes. The overlap between α-L-

### Table 2. Activities of glycosidase enzymes in livers from patients with HCC and from patients with cirrhosis

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Tumour tissue</th>
<th>Non-tumour tissue</th>
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<tr>
<td>(a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-L-Fucosidase</td>
<td>43 ± 22</td>
<td>87 ± 20</td>
</tr>
<tr>
<td>α-D-Mannosidase</td>
<td>15 ± 9</td>
<td>17 ± 14</td>
</tr>
<tr>
<td>α-Glucosidase</td>
<td>43 ± 27</td>
<td>49 ± 30</td>
</tr>
<tr>
<td>(b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-L-Fucosidase</td>
<td>13</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>54</td>
<td>60</td>
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<tr>
<td></td>
<td>28</td>
<td>42</td>
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<tr>
<td></td>
<td>11</td>
<td>11</td>
</tr>
</tbody>
</table>

Activities in (a) are mean ± SD values from both patients with HCC and patients with cirrhosis (n = 5 and n = 6, respectively) and those in (b) represent paired values from individual HCC patients (n = 4).
fucosidase activity in serum from patients with HCC and patients with cirrhosis suggests that this could arise in hepatocytes undergoing malignant or non-malignant change. Certainly, unusual protein fucosylation has been described in cirrhotic patients [25] and the levels of several fucose-containing proteins are markedly altered in Morris hepatoma cells relative to those found in normal rat liver [5]. Impaired fucose metabolism may be a feature of malignant and non-malignant hepatocyte regeneration.

ACKNOWLEDGMENT

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REFERENCES