Isolation of Carcinogen-induced Diploid Rat Hepatocytes by Centrifugal Elutriation

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ABSTRACT

The majority of hepatocytes isolated from rats treated with carcinogens (diethylnitrosamine plus 2-acetylaminofluorene) were found to be diploid, whereas most of the hepatocytes from normal rats are tetraploid. The carcinogen-induced diploid hepatocytes were only one-half the size (protein content) of the tetraploid hepatocytes, and could therefore be separated from the latter by centrifugal elutriation. The elutriation technique thus makes it possible to isolate a relatively pure fraction of carcinogen-induced cells. The diploid cells had the same liver-specific enzymatic and functional properties as the tetraploid cells and were thus undoubtedly of hepatic origin.

INTRODUCTION

Early stages of liver carcinogenesis in rodents are characterized by selective, clonal outgrowth of phenotypically altered (probably mutant) cell populations (1, 2). There is a tremendous phenotypic diversity among the altered cells, and changes in the expression of a variety of enzymes (1-4), iron storage deficiency (5), drug resistance (2), reduced numbers of asialo-glycoprotein receptors (6, 7) and a diminished autophagic activity (8) have been described. Although none of these "preneoplastic" traits have yet proved directly relevant to carcinogenesis, some attempts are currently being made to isolate potential cancer-precursor cell populations on the basis of their altered phenotype (4, 6).

We have recently reported that carcinogen treatment of rats induces a shift in the hepatocytic ploidy distribution from predominantly tetraploid to predominantly diploid (9). Since all persistent preneoplastic nodules which appear at a later stage are also found to be diploid (9), it seems highly probable that the relevant cancer-precursor cells are to be found within the diploid cell population. In the present report we show that the carcinogen-induced diploid cells are only one-half the size (protein content) of tetraploid cells and that they can therefore be separated from the latter by means of centrifugal elutriation (10).

MATERIALS AND METHODS

Animal Treatment. Four-week-old male Wistar rats from Møller-gaards Avlslaboratorium, Skovsved, Denmark were treated sequentially with the two carcinogens diethylnitrosamine and 2-AAF as described previously (8). Briefly, diethylnitrosamine (50 mg/kg body weight) was injected i.p. 24 h after a two-thirds partial hepatectomy. One week later the rats were fed a diet containing 0.02% 2-AAF. After 4 weeks on the 2-AAF diet, the rats were switched to a basal diet for 3 weeks before being used in experiments. Age-matched, untreated rats were used as controls. All animals were accustomed to the light/feeding schedule described previously (8).

Isolation and Elutriation of Cells. Liver cells were isolated by the two-step collagenase perfusion technique (11). After centrifugal removal of the nonparenchymal cells (4 washes and resedimentations of the hepatocytes at 300 rpm × 2 min in a Sorvall RC-3B centrifuge), the hepatocytes (final cell suspension) were suspended in a buffered, balanced salt solution (suspension buffer) containing 20 mM pyruvate. The buffer was supplemented with 1% bovine serum albumin when used in elutriation. The cells were filtered through a 63-μm nylon mesh to remove larger cell aggregates. Cells, 50-200 million (approximately, 10 million cells/ml) were introduced into the elutriation chamber of a JE-10X rotor (J6M centrifuge; Beckman) at a pump speed of 90 ml/min (Masterflex pump; Cole Palmer) and a centrifugal setting of 900 rpm at 4°C. After 10 min of stabilization in the chamber, 5 fractions were eluted by increasing the flow rate to 105, 120, 140, 170, and 200 ml/min, respectively. The elution volume for each fraction was 1800 ml. The eluted cells were sedimented (10 min at 700 rpm in a Sorvall 3C-B centrifuge with four 0.5-liter buckets) and resuspended in the pyruvate-containing buffer. The first fraction (flow rate, 105 ml/min) contained a few nonparenchymal cells in addition to small parenchymal cells, the viability of which varied between 50 and 85% (as determined by trypan blue exclusion (11)). In all other fractions the viability was approximately 95%.

Flow-cytometric Measurements of DNA and Protein Content. Single-parameter measurements were performed as described previously (9).

Electron Microscopy. The elutriated cell fractions were fixed by mixing two parts of cell suspension with one part 3% glutaraldehyde in 0.1 M cacodylate buffer. After rinsing overnight in 0.1 M cacodylate buffer, the samples were postfixed in osmium tetroxide and processed by standard procedures (12). Pictures were taken in a Siemens Elmecope 1 A operating at 80 kV, at a primary ×1600.

Enzyme Assays. β-N-Acetylgalactosaminidase was assayed as described by Barrett (13).

Acid phosphatase activity was determined essentially according to Barrett (13), using phenolphthalein monophosphate (1 mM) as a substrate in 0.15 M NaCl and 0.1 M citrate, pH 4.3. After 30 min at 37°C, the reaction was stopped by the addition of an equal volume of 0.5 M sodium carbonate, and the concentration of unesterified phenolphthalein was determined at 552 nm (extinction coefficient, 31 352 M-1 cm-1).

This substrate gives slightly higher activity values than 0.1 M /3-glycerophosphate.

The absorbance at 552 nm was plotted against time and the specific activity was calculated by the formula:

\[
\text{Specific activity} = \frac{A_552 \times 1000}{	ext{time} \times 2 	imes 10^{-3} \times 	ext{protein content}}
\]

where A552 is the absorbance at 552 nm, time is the time in minutes, and protein content is in mg/ml.

350 nm.5'Nucleotidase was measured according to EI-Aaser and Reid (15), using phenolphthalein monophosphate (1 HU) as a substrate in 0.15 M NaCl and 0.1 M citrate, pH 4.3. After 30 min at 37°C, the reaction was stopped by the addition of an equal volume of 0.5 M sodium carbonate, and the concentration of unesterified phenolphthalein was determined at 552 nm (extinction coefficient, 31 352 M-1 cm-1). This substrate gives slightly higher activity values than 0.1 M /3-glycerophosphate.

Cathespin B was assayed with N-benzoyl-arginine-2-naphthylamide as a substrate (13). The reaction was stopped and color development was initiated by adding 2 ml of ice-cold 0.05 M citric acid containing 2% Brij 35 and diazotized 2-methoxy-4-nitroaniline, 4 mg/ml ("Fast Red B salt"). The color was read at 540 nm.

Glutathione S-transferase was assayed by the method of Asaka and Takahashi (14), which is based on the determination of nitrite released from 1,2-dinitrobenzene in the reaction with glutathione. As the resulting color is read at 540 nm, the assay is less influenced by turbidity than other colorimetric assays, in which readings are made at around 350 nm.5'Nucleotidase was measured according to EI-Aaser and Reid (15), and arginase was assayed as described in (16). The enzyme activity in the reaction was stopped by the addition of an equal volume of 0.5 M sodium carbonate, and the concentration of unesterified phenolphthalein was determined at 552 nm (extinction coefficienct, 31 352 M-1 cm-1).

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1 Supported by The Norwegian Cancer Society.
2 Fellow of The Norwegian Cancer Society.
3 The abbreviations used are: 2-AAF, 2-acetylaminofluorene; GGT, γ-glutamyl transpeptidase.

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ISOLATION OF PRENEOPLASTIC HEPATOCYTES BY ELUTRIATION

RESULTS AND DISCUSSION

Nonselective Purification of Isolated Hepatocytes. The collagenase perfusion method results in a complete dissociation of the liver, i.e., the yield of hepatocytes in the initial dissociate is virtually quantitative. This initial cell suspension also contains 10–15% nonparenchymal cells, a number that can be reduced by using differential centrifugation to purify the hepatocytes (11). In the present study, we chose centrifugation conditions which gave a high yield of purified hepatocytes (45%) at the expense of a relatively moderate purification (approximately, 2-fold; cf. Table 1) in order to ensure that the final hepatocyte suspension was representative of the whole liver. This was further verified by comparing enzymatic and cellular properties of the initial and final cell suspension (Table 1). The ploidy distributions were not significantly altered, the high percentage of the initial and final cell suspension (Table 1). The ploidy classes was not achieved (21). The ploidy classes was not achieved (21).

Size and Ploidy Distributions of Isolated Hepatocytes. Table 1 shows that the majority of hepatocytes from carcigen-treated livers are diploid. The diploid percentage is actually higher than indicated in the table: if correction is made for 2N-tetraploid hepatocytes, the overall viability in this fraction was 50–75% of the hepatocytes are found to be diploid (9). In contrast, only about 10% of the hepatocytes in normal livers are diploid (9). By 2-parameter flow cytometry it can be demonstrated that diploid cells are, on average, one-half the size of tetraploid cells both in normal and carcigen-treated rats (19).

Separation of Small (Diploid) and Large (Tetraploid) Hepatocytes by Centrifugal Elutriation. The centrifugal elutriation technique separates cells according to sedimentation rate, i.e., largely according to size (10), and should therefore be suitable for the separation of diploid and tetraploid hepatocytes. Size separation by elutriation has previously been reported with hepatocytes from both normal and carcigen-treated rats (6, 20, 21), but good resolution of the different ploidy classes was not achieved (21). Fig. 1 shows DNA histograms of an unfractionated hepatocyte suspension from a carcigen-treated rat (A) and of five elutriated fractions from the same suspension (B–F), collected with hepatocytes from both normal and carcigen-treated rats (6, 20, 21), but good resolution of the different ploidy classes was not achieved (21).
Fig. 2. Protein histograms of elutriated fractions of hepatocytes from a carcinogen-treated rat. A, total suspension; B and C, elutriated fractions 2 and 4.

Fig. 2 displays protein histograms of these two fractions as well as of the whole suspension, and it can be seen that the diploid cells from fraction 2 (Fig. 2B) had approximately one-half the size (protein content) of the cells from fraction 4 (Fig. 2C). Light micrographs (Fig. 3) likewise show that the diploid cells of fraction 2 (Fig. 3B) were considerably smaller than the tetraploid cells of fraction 4 (Fig. 3C). The cells in fractions 2 and 4 had mean diameters of 17.3 ± 0.1 (SE) and 22.6 ± 0.2 μm, respectively, as measured with a ruled eyepiece (~200 cells measured in each fraction). These values, corresponding to volumes of ~2700 and ~6000 μm³, respectively, agree well with size determinations performed by Bernaert et al. (20) on elutriated fractions from normal hepatocyte suspensions. The cells in the total (unfractionated) suspension (Fig. 3A) exhibited the expected size heterogeneity.

Morphological and Functional Characterization of Diploid and Tetraploid Hepatocytes. To verify that the diploid cells isolated by elutriation really were hepatocytes and not some other hepatic cell type, they were evaluated by a number of morphological and biochemical criteria and compared to the tetraploid cells. In the light microscope, both cell types had the characteristic appearance and yellow-brown color of hepatocytes, not seen in any other kind of cell in the liver (8). The diploid cells were furthermore twice as large as the (colorless) cells of bile duct lineage like oval, transitional, and bile ductular cells (22, 23), cell types that are hardly present in detectable numbers in these cell suspensions (8). The sheer number of diploid cells, corresponding to about one-half of the liver weight (9) makes it obvious that they are hepatocytes.

Both the diploid and tetraploid fraction of elutriated hepatocytes (fractions 2 and 4, respectively) displayed a completely normal hepatocytic ultrastructure (Fig. 4). Apart from size, the two cell types were indistinguishable in the electron microscope.
One of the most unique biochemical functions of the hepatocyte is glucogenesis, i.e., the ability to synthesize and release glucose. The glucogenic capacity was tested in both diploid and tetraploid fractions, incubated as intact cells for 2 h at 37°C. The cells were given 10 mM lactate as a gluconeogenic substrate. The rate of glucose formation under such conditions will be the sum of gluconeogenesis (mainly from lactate) and glycogen degradation, the latter expected to be moderate since the cells were prepared from starved rats (18).

The two cell classes displayed the same glucogenic rate (Table 2), proving that they both represent bona fide hepatocytes, and that they are functionally equivalent. The rate of glucogenesis was of the same order as in normal hepatocytes under similar conditions (18). The alterations in carbohydrate metabolism, particularly the glucose 6-phosphatase deficiency frequently associated with preneoplastic lesions (1, 3) are thus apparently not of a sufficient magnitude to significantly affect the overall function of the hepatocyte population at this stage of liver carcinogenesis.

The cell surface receptor for asialoglycoproteins is another hepatocyte-specific marker that was present in equal amounts (in relation to protein content) in diploid and tetraploid cells (Table 2). The overall concentration of this receptor was markedly reduced in carcinogen-treated animals versus controls (6, 7), and it is interesting that this reduction apparently affected the whole hepatocyte population in a uniform manner.

Hepatocytes from carcinogen-treated rats have previously been found to exhibit reduced rates of autophagic sequestration and autophagic-lysosomal protein degradation (8). As shown in Table 2, diploid and tetraploid cells showed the same low rate of protein degradation, i.e., their autophagic activity was apparently suppressed to the same extent. Besides additionally documenting the functional equivalence of the two ploidy classes, these results suggest that the suppression of autophagy, like the reduction in asialoglycoprotein receptors, affects the hepatocyte population uniformly. Effects of this kind could reflect regulatory mechanisms, whereby carcinogen-induced al-
ISOLATION OF PRENEOPLASTIC HEPATOCYTES BY ELUTRIATION

Table 2 Enzymatic and functional properties of diploid and tetraploid hepatocytes isolated from carcinogen-treated rats

<table>
<thead>
<tr>
<th></th>
<th>Total cell suspension</th>
<th>Diploid hepatocytes</th>
<th>Tetraploid hepatocytes</th>
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<tbody>
<tr>
<td>Functional properties</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucogenesis (µg glucose/ mg protein)</td>
<td>30.8 (1)</td>
<td>28.7 (1)</td>
<td>30.1 (1)</td>
</tr>
<tr>
<td>Asialo-orsomucoid binding (µmol/mg protein)</td>
<td>273 ± 40 (4)</td>
<td>230 ± 47 (4)</td>
<td>254 ± 41 (4)</td>
</tr>
<tr>
<td>Protein degradation (%)</td>
<td>3.1 ± 0.4 (5)</td>
<td>3.1 ± 0.4 (5)</td>
<td>3.3 ± 0.5 (5)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzyme activities (milliunits/mg protein)</th>
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<tbody>
<tr>
<td>Acid phosphatase</td>
<td>24.7 ± 1.3 (4)</td>
<td>28.5 ± 2.6 (4)</td>
<td>24.7 ± 1.3 (4)</td>
</tr>
<tr>
<td>β-Acetethylglucosaminidase</td>
<td>9.8 ± 0.9 (9)</td>
<td>11.1 ± 0.4 (4)</td>
<td>10.6 ± 0.4 (4)</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>1.0 ± 0.04 (4)</td>
<td>0.9 ± 0.04 (4)</td>
<td>0.9 ± 0.04 (4)</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>13.3 ± 1.6 (4)</td>
<td>12.6 ± 0.4 (4)</td>
<td>13.2 ± 2.2 (4)</td>
</tr>
<tr>
<td>Reduced glutathione transferase</td>
<td>29.6 ± 1.9 (9)</td>
<td>31.2 ± 4.9 (2)</td>
<td>28.2 ± 1.7 (4)</td>
</tr>
<tr>
<td>Arginase</td>
<td>7.8 ± 0.5 (11)</td>
<td>9.1 ± 0.7 (5)</td>
<td>9.3 ± 1.0 (5)</td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>15.7 ± 1.2 (9)</td>
<td>13.8 ± 0.9 (4)</td>
<td>15.3 ± 1.4 (4)</td>
</tr>
<tr>
<td>γ-Glutamyl transpeptidase</td>
<td>24.5 ± 0.7 (3)</td>
<td>35.2 ± 0.2 (3)</td>
<td>15.2 ± 0.8 (3)</td>
</tr>
<tr>
<td>(% positive cells)</td>
<td></td>
<td></td>
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</table>

- Glucogenesis was measured as the rate of glucose production by intact hepatocytes, incubated with 10 mM lactate for 2 h at 37°C.
- Numbers in parentheses, number of cell preparations.
- Asialo-orsomucoid binding was measured as in Ref. 7.
- Mean ± SE.
- Degradation of long-lived protein, measured as in Ref. 8.
- Enzyme activities were measured in homogenates from unfractionated hepatocyte suspensions or from diploid/tetraploid hepatocytes isolated by centrifugal elutriation.
- Activities nonspecifically measured; i.e., several types of reduced glutathione transferase are included.

A minority of the cells would cause secondary changes in all of the cells. A number of enzymes were measured in diploid and tetraploid hepatocyte fractions as well as in the total (unfractionated) cell suspension (Table 2). Enzyme activities were expressed on a “per protein” basis in order to correct for the cell size difference. With the exception of reduced glutathione transferase, which was 40% higher, and GGT (see below), all enzymes tested had the same activity in cells from normal and carcinogen-treated rats.

Three lysosomal enzymes (acid phosphatase, β-acetethylglucosaminidase, and cathepsin B), one mitochondrial enzyme (cytochrome c oxidase), one plasma membrane enzyme (5′-nucleotidase), and two predominantly cytosolic enzymes (glutathione S-transferase and arginase) all showed similar specific activities in diploid and tetraploid cells as well as in the total cell suspension. Cells of different ploidies thus seem to be functionally equivalent, both in terms of the “household” functions represented by most of these enzymes and with respect to the hepatocyte-specific urea cycle enzyme, arginase. Enzymatic similarity also seems to be the rule in diploid and tetraploid hepatocytes from normal liver.

However, in one respect the two ploidy classes were different. The number of GGT-positive cells, 10-fold increased by the carcinogen treatment (results not shown), was more than twice as high in the diploid cell class (Table 2). Although GGT is not necessarily directly relevant to carcinogenesis, it may serve as a marker of carcinogen-induced changes in the cells. This finding thus may be interpreted in favor of our hypothesis that the diploid hepatocyte class is enriched in carcinogen-induced cells (9).

We have previously estimated that about 95% of the diploid hepatocytes in carcinogen-treated rats may have been specifically induced by the carcinogen treatment (9). The diploid cell fraction isolated by means of the elutriation technique can therefore be regarded as a highly purified preparation of carcinogen-induced cells. The availability of such cells in large quantity may allow systematic investigation of their carcinogenic potential, e.g., by means of transplantation (26). Experiments involving transplantation of hepatocytic subpopulations are now being performed in several laboratories (27, 28), and will hopefully help to characterize the early stages of liver carcinogenesis.

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