Digitonin–collagenase perfusion for efficient separation of periportal or perivenous hepatocytes

Kai O. LINDROS and Kai E. PENTTILÄ
Research Laboratories of the State Alcohol Company, Alko Ltd., POB 350, SF-00101 Helsinki, Finland

(Received 13 February 1985/21 March 1985; accepted 11 April 1985)

Intact rat liver cells from the perivenous region were isolated by collagenase perfusion after first destroying the periportal region by a brief portal infusion of digitonin. Periportal cells were isolated after retrograde digitonin infusion. Significantly higher alanine aminotransferase, γ-glutamyltransferase and lactate dehydrogenase activities and lower glutamate dehydrogenase and pyruvate kinase activities in periportal than in perivenous cells demonstrate marked separation. The high yield allows further characterization in vitro of the cell populations.

Metabolic heterogeneity of the acinus, the microcirculatory unit of the liver (Rappaport et al., 1954), was first demonstrated by histochemistry. More recently, the acinar distributions of various enzymes was semi-quantified with a microdissection technique followed by biochemical analysis (Jungermann & Katz, 1982). Studies aiming to separate intact periportal zone-1 hepatocytes from perivenous zone-3 cells, in order to study the basis for and the consequences of this metabolic zonation (Jungermann & Katz, 1982; Gumucio & Miller, 1981), have usually failed to present convincing evidence that the separated subfractions originated from different acinar regions (Weigand et al., 1974; Wanson et al., 1975; Gumucio et al., 1978; Sumner et al., 1983). Partial separation has been achieved by careful high-resolution density-gradient centrifugation (Bengtsson et al., 1981). Better separation, but with a lower and more variable yield, was obtained by a method developed in our laboratory based on the principle of local collagenase digestion (Vännänen et al., 1983). We have now developed a high-yield method based on initial selective destruction of one acinar region, followed by isolation of the cells from the intact part. Quistorff et al. (1985) had observed that selective leakage of periportal or perivenous cellular contents could be obtained by ante- or retro-grade infusion of digitonin, a cholesterol-complexing agent previously used to destroy plasma membranes for isolation of subcellular organelles (Janski & Cornell, 1980; Zuurendonk & Tager, 1974). We have combined a modification of this digitonin procedure with subsequent collagenase digestion for high-yield production of periportal or perivenously enriched hepatocytes.

Materials and methods

Animals

Male rats of the Alko mixed strain (7 weeks old) weighing around 200 g and fed on standard laboratory diet (Astra-Ewos AB, Södertälje, Sweden) and tap water ad libitum were used.

Digitonin–collagenase perfusion

After anaesthesia (60 mg of sodium pentobarbital/kg body wt., intraperitoneally), the liver was pre-perfused at 37°C and about 40 ml/min in situ without recirculation via the portal vein with buffer A (137 mM-NaCl, 4.7 mM-KCl, 1.1 mM-CaCl₂, 0.65 mM-MgSO₄, 5.6 mM-glucose and 10 mM-Hepes, pH 7.4 at room temperature) oxygenated in a silastic-tubing oxygenator (Hamilton et al., 1974). The papilliform lobe was then removed and put into liquid N₂, and the vena cava superior was cannulated.

A 7 mM digitonin solution (AnalyR; BDH Chemicals, Poole, Dorset, U.K.), prepared in buffer A at 100°C, was infused at 40°C through either cannula at a rate of 10 ml/min for 25–45 s. The infusion was stopped when the pattern on the liver surface characteristic of selective destruction was maximal (see Fig. 1).

Perfusion with Ca²⁺-free buffer (142 mM-NaCl, 6.7 mM-KCl, 5.6 mM-glucose and 10 mM-Hepes, pH 7.55) was initiated through the opposite cannula 10–20 s after stopping the infusion of digitonin, and continued for 10 min (Seglen, 1976). A conventional
collagenase perfusion (Berry & Friend, 1969) was then started from the direction opposite to the digonin pulse by air-equilibrated buffer (68 mM-NaCl, 6.7 mM-KCl, 4.1 mM-CaCl₂, 5.6 mM-glucose and 100 mM-Hepe, pH 7.70) supplemented with 0.5 mg of collagenase/ml and 15 mg of fatty acid-free albumin/ml (both from Boehringer, Mannheim, Germany) at 10 ml/min for 9–13 min. Finally the collagenase-digested liver was briefly flushed with 10–15 ml of buffer A.

**Purification of hepatocytes**

The liver was removed, and the cells were released by gentle combing into 50 ml of buffer B [buffer A plus 2% (w/v) albumin] with 20 µg of deoxyribonuclease/ml on a Petri dish. The suspension was filtered through Monyl nylon mesh (160 and 61 µm; Zürich Bolting Cloth Manufacturing Co., Zürich, Switzerland), and incubated at room temperature in a closed 500 ml Erlenmeyer flask under an O₂ atmosphere for 10 min with gentle stirring. The cells were purified by pelleting twice (15g max. for 90s) from 50 ml of buffer B in a 100 ml polycarbonate tube. If the viability of the initial cell suspension was below 70%, the first pellet was suspended in 25 ml of cooled 30% (w/v) Metrizamide buffer [2.5 mM-NaCl, 4.0 mM-KCl, 0.92 mM-CaCl₂, 0.55 mM-MgSO₄, 4.7 mM-glucose and 8.6 mM-Hepe, 0.85% albumin and 30% (w/v) Metrizamide (analytical grade; Nyegaard & Co. A/S, Oslo, Norway), pH 7.45], and 5 ml of buffer B was layered on top (Seglen, 1976). After centrifugation for 3 min at 27g max., intact hepatocytes were harvested by Pasteur pipette at the phase boundary and washed once by centrifugation with 50 ml of cooled buffer B (1 min, 15g max.).

The final pellet was suspended in buffer B; batches were frozen in liquid N₂ and stored at −80°C.

Viability was determined by dye exclusion with 0.05% eosin, and the yield from cell concentration was determined by the hepatocrit method (Bengtsson et al., 1981).

**Biochemical determinations**

The cell pellet was treated with 1% Triton X-100. Protein was assayed by the fluorescamine method (Böhlen et al., 1973).

Standard u.v. methods were used for assays of alanine aminotransferase (EC 2.6.1.2) (Scandinavian Committee on Enzymes, 1974), glutamate dehydrogenase (EC 1.4.1.2) and lactate dehydrogenase (EC 1.1.1.27) (Bergmeyer, 1970). For pyruvate kinase (EC 2.7.1.40) the method for serum (Bergmeyer, 1970) was used, except that 0.1 mM-fructose 1,6-bisphosphate (Llorente et al., 1970) was present. γ-Glutamyltransferase (EC 2.3.2.2) was determined colorimetrically (Scandinavian Committee on Enzymes, 1976) with a test kit (Medix Biochemica, Kauniainen, Finland).

**Results**

**Effect of digitonin**

The rupturing of membranes by digitonin could be seen as a regional bleaching within 10–20 s. A pale network indicating destroyed perportal areas appeared when the digitonin had been infused via the portal vein (Fig. 1a); the network pattern usually reached its maximal intensity in about 25 s, when 4–5 ml of digitonin solution had been infused. When digitonin was infused via the hepatic veins a complementary pattern of pale spots appeared, indicating cell destruction of the perivenous area (Fig. 1b). The decolorization

---

**Fig. 1. Area-specific cell destruction after digitonin infusion**

(a) Decolorization of peribortal areas (appearing as the pale network) after infusion of digitonin via the portal vein. (b) Decolorization of the perivenous areas (appearing as light spots) after infusion of digitonin via the hepatic veins. Magnification approx. ×2.5.
generally took slightly longer to appear when retrograde digitonin perfusion was applied.

**Separation of intact cells**

The initial cell suspension obtained after the conventional collagenase perfusion contained much cell debris. The initial cell viability varied considerably, but was commonly above 70%. After purification of the cells by centrifugations, or (if the initial viability was below 70%) by Metrizamide, a final suspension was obtained with a viability generally exceeding 90%.

The yield of purified cells from the periportal region was almost twice (1870 ± 620 mg of packed cells; mean ± s.d., n = 6) that from the perivenous region (990 ± 290 mg; n = 6). When the initial cell viability was low and cells had to be purified with Metrizamide, the final yield was much lower.

**Marker enzyme activities**

Five marker enzymes were analysed both from the purified cells and from the papilliform lobe ligated before digitonin treatment, in order to evaluate the selectivity of the procedure (Table 1). The activities of alanine aminotransferase, lactate dehydrogenase and γ-glutamyltransferase are higher in the portal cells, whereas glutamate dehydrogenase and pyruvate kinase activities are higher in the perivenous cells. The activities are expressed both per mg of protein and as the relative activity compared with that found in the corresponding liver lobe. The periportal/perivenous ratios of the enzymes are essentially similar regardless of how the activities are expressed, but the biological variation is decreased by using the cell/liver ratio, as indicated by the smallest standard deviations with these measures. Statistically significant differences between periportal and perivenous cell preparations were found for all five enzymes. The periportal dominance of alanine aminotransferase and lactate dehydrogenase and the perivenous dominance of glutamate dehydrogenase and pyruvate kinase have been observed in several earlier studies using either microdissection (Jungermann & Katz, 1982) or cell-separation techniques (Bengtsson et al., 1981; Väänänen et al., 1984). The possible implications of the recently observed marked periportal dominance of γ-glutamyltransferase (H. Speisky, Y. Israel & K. Lindros, unpublished work) are not discussed here.

**Discussion**

This study demonstrates that a population of cells enriched from one acinar region can be obtained by selectively destroying cells from the opposite region. The selectivity of the digitonin damage was confirmed from the differences in marker enzyme activities observed in the effluents collected after ante- or retro-grade digitonin infusion (results not shown).

The time course for the development of the decolorization pattern was quite reproducible. When 7 mM-digitonin was infused at 10 ml/min, the ratio [optimal pulse length (s)/rat body wt. (g)] was 0.10–0.14 for portal and 0.15–0.22 for retrograde infusion. The dead space of the vena cava at least partly explains the larger volume required for retrograde digitonin infusion. Rough calculations based on a mean content of 8 μmol of cholesterol/g of liver indicate that the infused digitonin could complex quantitatively with all available cholesterol in the affected region. Digitonin apparently destroys many cells to fragments, thus explaining the commonly high initial cell viability even after widespread decolorization of the liver surface. The larger yield of portal hepatocytes was probably associated with less enrichment, as indicated by the smaller enzyme-activity differences between cell and liver samples (Table 1). Attempts to increase selectivity by prolonging the digitonin infusion usually resulted in low initial viability and in difficulties in separating the dye-excluding cells.

We conclude that the reproducible and marked

---

**Table 1. Marker enzyme activities in hepatocytes isolated from the periportal or the perivenous region**

Mean values ± s.d. of six preparations of each group are given. All activities are expressed as nmol/min per mg of protein. The cell/liver ratio denotes the cell activity relative to that observed in a sample from the corresponding liver.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Periportal</th>
<th>Perivenous</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Activity</strong></td>
<td><strong>Cell/liver ratio</strong></td>
<td><strong>Cell/liver ratio</strong></td>
</tr>
<tr>
<td>Alanine aminotransferase</td>
<td>245 ± 57</td>
<td>1.73 ± 0.20</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>2010 ± 230</td>
<td>1.29 ± 0.07</td>
</tr>
<tr>
<td>γ-Glutamyltransferase</td>
<td>2.44 ± 1.02</td>
<td>1.18 ± 0.42</td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>1100 ± 170</td>
<td>1.08 ± 0.06</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>202 ± 38</td>
<td>0.97 ± 0.09</td>
</tr>
</tbody>
</table>
separation of periportal and perivenous cell populations by this method should facilitate further studies in vitro on the basis for and consequences of the metabolic heterogeneity of the liver acinus.

References