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

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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 microbiochemical 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 (Väänänen *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 periportally or perivenously enriched hepatocytes.

Materials and methods

Animals

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

Digitonin–collagenase perfusion

After anaesthesia (60mg of sodium pentobarbital/kg body wt., intraperitoneally), the liver was pre-perfused at 37°C and about 40ml/min *in situ* without recirculation via the portal vein with buffer A (137mM-NaCl, 4.7mM-KCl, 1.1mM-$CaCl_2$, 0.65mM-$MgSO_4$, 5.6mM-glucose and 10mM-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 N_2, and the vena cava superior was cannulated.

A 7mM digitonin solution (AnalaR; 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 10ml/min for 25–45s. The infusion was stopped when the pattern on the liver surface characteristic of selective destruction was maximal (see Fig. 1).

Perfusion with Ca^{2+}-free buffer (142mM-NaCl, 6.7mM-KCl, 5.6mM-glucose and 10mM-Hepes, pH 7.55) was initiated through the opposite cannula 10–20s after stopping the infusion of digitonin, and continued for 10min (Seglen, 1976). A conventional

collagenase perfusion (Berry & Friend, 1969) was then started from the direction opposite to the digitonin pulse by air-equilibrated buffer (68mM-NaCl, 6.7mM-KCl, 4.1mM-$CaCl_2$, 5.6mM-glucose and 100mM-Hepes, pH7.70) supplemented with 0.5mg of collagenase/ml and 15mg of fatty acid-free albumin/ml (both from Boehringer, Mannheim, Germany) at 10ml/min for 9–13min. 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 50ml of buffer B [buffer A plus 2% (w/v) albumin] with 20μg of deoxyribonuclease/ml on a Petri dish. The suspension was filtered through Monyl nylon mesh (160 and 61μm; Zürich Bolting Cloth Manufacturing Co., Zürich, Switzerland), and incubated at room temperature in a closed 500ml Erlenmeyer flask under an O_2 atmosphere for 10min with gentle stirring. The cells were purified by pelleting twice ($15g_{max.}$ for 90s) from 50ml of buffer B in a 100ml polycarbonate tube. If the viability of the initial cell suspension was below 70%, the first pellet was suspended in 25ml of cooled 30% (w/v) Metrizamide buffer [2.5mM-NaCl, 4.0mM-KCl, 0.92mM-$CaCl_2$, 0.55mM-$MgSO_4$, 4.7mM-glucose and 8.6mM-Hepes, 0.85% albumin and 30% (w/v) Metrizamide (analytical grade; Nyegaard & Co. A/S, Oslo, Norway), pH7.45], and 5ml of buffer B was layered on top (Seglen, 1976). After centifugation for 3min at $27g_{max.}$, intact hepatocytes were harvested by Pasteur pipette at the phase boundary and washed once by centrifugation with 50ml of cooled buffer B (1min, $15g_{max.}$).

The final pellet was suspended in buffer B; batches were frozen in liquid N_2 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 0.1mM-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 10–20s. A pale network indicating destroyed periportal areas appeared when the digitonin had been infused via the portal vein (Fig. 1*a*); the network pattern usually reached its maximal intensity in about 25s, when 4–5ml 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. 1*b*). The decolorization

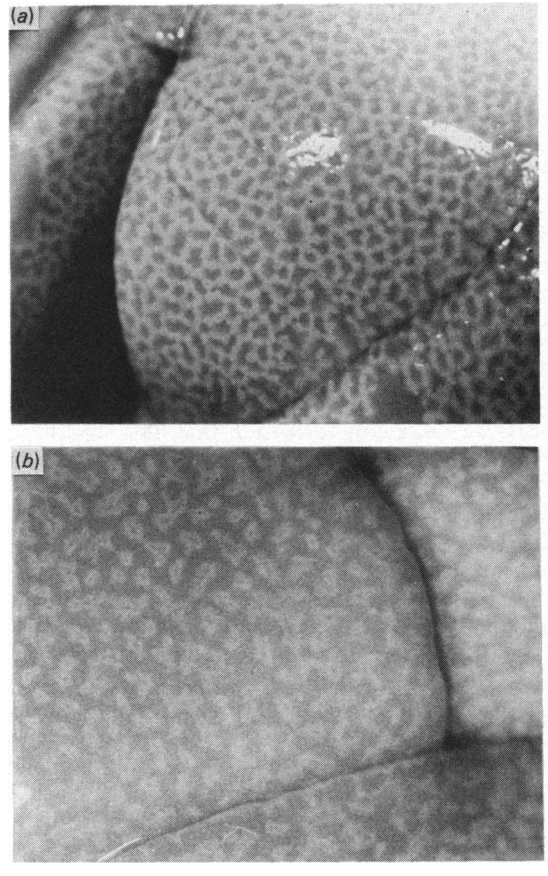

Fig. 1. *Area-specific cell destruction after digitonin infusion*
(*a*) Decolorization of periportal areas (appearing as the pale network) after infusion of digitonin via the portal vein. (*b*) Decolorization of the perivenous areas (appearing as light spots) after infusion of digitonin via the hepatic 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 ± 620mg of packed cells; mean ± S.D., $n = 6$) that from the perivenous region (990 ± 290mg; $n = 6$). 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 7mM-digitonin was infused at 10ml/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: $^{*}P < 0.025$, $^{**}P < 0.005$ and $^{***}P < 0.001$ for difference in cell/liver ratio between periportal and perivenous preparations.

	Periportal		Perivenous		
	Activity	Cell/liver ratio	Activity	Cell/liver ratio	Periportal/perivenous ratio
Alanine aminotransferase	245 ± 57	1.73 ± 0.20	116 ± 50	0.81 ± 0.16***	2.14
Lactate dehydrogenase	2010 ± 230	1.29 ± 0.07	1510 ± 130	0.98 ± 0.04***	1.32
γ-Glutamyltransferase	2.44 ± 1.02	1.18 ± 0.42	0.57 ± 0.14	0.35 ± 0.12**	3.42
Glutamate dehydrogenase	1100 ± 170	1.08 ± 0.06	1550 ± 70	1.49 ± 0.13***	0.72
Pyruvate kinase	202 ± 38	0.97 ± 0.09	241 ± 24	1.19 ± 0.15*	0.82

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.

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