Isolation and Primary Culture of Bovine Hepatocytes: Albumin Synthesis and Adrenergic Activation of Glycogenolysis

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ABSTRACT. We describe a technique for isolation and primary culture of bovine hepatocytes, and their metabolic characterization. Hepatocytes were isolated from the caudate lobe of bovine liver by perfusion with 0.25 mM ethyleneglycol tetracetic acid and 0.05% collagenase. The viability and yield of the cells were 70-92% and 0.1-3.6 × 10^7 cells/g liver, respectively. When the isolated hepatocytes were cultured in Williams' medium E, they began to spread in 3 hr and formed monolayers in 24 hr. These monolayers were retained for at least 6 days. To monitor the metabolic activities specific to liver, synthesis and secretion of albumin were measured by labeling with [35S]-methionine and immunoprecipitation. This activity was low in isolated hepatocytes, but increased after culturing 1-3 days, and decreased again after 6 days. Glycogenolytic activity was also assessed by measuring glucose release to the medium by stimulation with epinephrine. The glycogenolytic response to epinephrine was also enhanced by culturing the hepatocytes 1-3 days, but was decreased after 6 days. Since the isolated bovine hepatocytes retained the liver-specific activities of albumin synthesis and glycogenolysis for several days in culture, these cells are useful for cellular and molecular studies on the functions of bovine liver. —KEY WORDS: albumin synthesis, bovine, glycogenolysis, isolated hepatocyte, primary cultured hepatocyte.


It is well recognized that primary cultured hepatocytes are quite useful as a simple in vitro system for the studies on a wide variety of liver functions. Since the method for isolation and primary culture of hepatocytes was established first in rats, most informations have come from rodent species. However, there are apparent species differences in some functions of the liver. Particularly, the liver of ruminants has lots of metabolic activities different from those of other species. For instance, a primary substrate for gluconeogenesis is propionate more than lactate or amino acids [3, 4]. In addition, the synthesis and secretion of some proteins such as acute phase proteins seem to be regulated by a unique system(s) in bovine liver [14].

There have been some reports on isolation and culture of bovine hepatocytes [8, 17, 19]. Shull et al. [17] first described a technique for isolation and culture of bovine hepatocytes. These authors, however, observed detachment of the cells and a rapid decrease in drug-metabolizing activity after 8 hr in culture. Recently Van’t Klooster et al. [19] also examined the similar activity of cultured bovine and goat hepatocytes, but found again a drop in the activity after 51 hr in culture. However, drug-metabolizing activity may be less appropriate as a marker of the metabolic functions of hepatocytes in vitro, because its rapid loss was also found in cultured rat hepatocytes retaining other activities specific to liver [5, 18]. Thus, it remains to be clarified whether the primary cultured ruminant hepatocytes can maintain the activity of liver-specific functions and can be used for biochemical studies. In this report, we describe a technique for isolation and primary culture of bovine hepatocytes. As the liver-specific functions, we investigated albumin synthesis and glycogenolytic response for 6 days in culture.

MATERIALS AND METHODS

Animals and chemicals: Three healthy calves listed in Table 1 were used.

The following chemicals were purchased: xylazine, tryptophan inhibitor, aprotinin, insulin, epinephrine (Sigma, St. Louis, MO, U. S. A.); collagenase (228 U/mg), dexamethasone, ethyleneglycol tetracetic acid (EGTA) (Wako, Tokyo); pentobarbital sodium (Abbott Laboratories, North Chicago, IL, U. S. A.); heparin (Kodama, Tokyo); Williams' medium E (WE), methionine-free Dulbecco's modified Eagle's medium (DME) (Gibco, Grand Island, NY, U. S. A.); fetal calf serum (FCS) (Flow Laboratories, McLean, VA, U. S. A.); streptomycin, penicillin, kanamycin (Meiji, Tokyo); fungizone (Bristol-Myers Squibb, Tokyo); N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), phenylmethylsulfonfyl fluoride (PMSF) (Nacalai Tesque, Kyoto); L-[35S]-methionine (>37.0 TBq/mmol) (DuPont, Boston, MA, U. S. A.); rabbit anti-bovine albumin antisera (Daco Japan, Kyoto); protein A (Genzyme, Cambridge, MA, U. S. A.); CNBr-activated Sepharose 4B (Pharmacia-LKB Biotechnology, Uppsala, Sweden); leupeptin, pepstatin (Peptide Institute, Osaka); prestained SDS-PAGE standards (BioRad, Richmond, CA, U. S. A.).

Collagen was prepared from rat tail tendon according to the method of Elsdale and Bard [7].

Isolation of hepatocytes: The caudate lobe of the liver was removed by cutting across its base of attachment following euthanasia or anesthesia by xylazine (0.15
Table 1. Characteristics of the animals, cell yield and viability of isolated bovine hepatocytes

<table>
<thead>
<tr>
<th>Strain</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Holstein</td>
<td>Male</td>
<td>Holstein</td>
</tr>
<tr>
<td>Age (month)</td>
<td>5</td>
<td>60</td>
<td>150</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>92</td>
<td>64</td>
<td>160</td>
</tr>
<tr>
<td>Cell viability (%)</td>
<td>92</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Cell yield (×10⁷ cells/g liver)</td>
<td>3.6</td>
<td>0.8</td>
<td>0.1</td>
</tr>
<tr>
<td>Total cell yield (×10⁸ cells)</td>
<td>33</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

mg/kg, i. m.) and pentobarbital sodium (16 mg/kg, i. v.). Immediately after removal, blood of the tissue was washed out by perfusing the major blood vessels of the lobe with ice-cold saline containing 500 U/ml of heparin at a flow rate of 50 ml/min using a 50 ml/plastic syringe (Terumo, Tokyo) and a pump (Cole Parmer Instrument, Chicago, IL, U. S. A.). The flushed lobe was kept in ice-cold phosphate buffered saline (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, pH 7.4).

The procedure for the isolation of hepatocytes was the modification of the method of Seglen [16]. The liver lobe was placed in an autoclavable bag (750 x 620 mm) (Iwaki Glass, Osaka) and perfused from the major blood vessels at the rate of 50 ml/min using a syringe and a pump (Fig. 1). All perfusion buffers were sterile, pH 7.5, 37°C and saturated with 5%CO₂-95%O₂. The lobe was perfused first with 500 ml of perfusion buffer A (0.14 M NaCl, 5.4 mM KCl, 0.5 mM Na₂HPO₄, 0.4 mM Na₂HPO₄, 10 mM HEPES, 4 mM NaHCO₃) containing 0.25 mM EGTA and 5 mM glucose. The second perfusion buffer was 300 ml of Ca²⁺-free Hanks’ solution (0.14 M NaCl, 5.4 mM KCl, 0.33 mM Na₂HPO₄, 4 mM NaHCO₃, 5.5 mM glucose, 0.4 mM KH₂PO₄, 0.4 mM MgSO₄). Then the third perfusion buffer was 300 ml of perfusion buffer A containing 0.05% collagenase, 5 mM CaCl₂, 0.05 mg/ml trypsin inhibitor and 0.12 µg/ml aprotinin. The third buffer was re circulated through the lobe for 10-15 min. After perfusion the lobe was transferred to a glass dish (25 cm), trimmed to remove non-perfused area, and cut into 10 mm pieces with surgical scalpels. Hepatocytes were dispersed in 200 ml of Ca²⁺-free Hanks’ solution by gently shaking in a glass flask for 5 min at 37°C. After sieving through a nylon mesh (0.125 mm), cells were washed by centrifugation (50 x g, 3 min) in Ca²⁺-free Hanks’ solution three times. The resulting cell pellet was suspended in a culture medium to give a cell density of 5 x 10⁵ cells/ml.

Primary culture of hepatocytes: The culture medium was WE supplemented with 5% FCS, 1 µM insulin, 10 µM dexamethasone, 50 U/ml penicillin, 50 µg/ml streptomycin, 0.25 µg/ml fungizone, 100 µg/ml kanamycin, 1 mM CaCl₂, and 1 mM MgCl₂. Aliquots of 0.3 ml and 2 ml cell suspensions were seeded into 16 mm and 35 mm plastic tissue-culture dishes (Nunc, Roskilde, Denmark) coated with rat collagen for the measurements of albumin synthesis and glycogenolysis, respectively. The dishes were incubated at 37°C in a humid atmosphere of 5% CO₂ in air. After incubating for 3 hr the medium was replaced by that not containing CaCl₂ and MgCl₂. Medium change was carried out after the first 24 hr of culture and every 2 days thereafter.

Synthesis and secretion of albumin: Synthesis and secretion of albumin were measured according to the procedure of Andus et al. [1] with some modifications. [¹⁵S]-methionine (300 kBq) was added to 0.3 ml methionine-free DME culture medium. After incubation at 37°C for 2 hr, 0.3 ml medium was separated from the cells and centrifuged at 12,000 x g for 5 min. The proteins in the medium were precipitated with 5% trichloroacetic acid (TCA) containing 30 mg of carrier skim milk (Morinaga, Tokyo). The precipitate was washed three times with 5% TCA, and solubilized in 1 ml of solution A (0.1 M Tris-HCl, pH 7.6, 0.14 M NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS)) containing 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml pepstatin, and 10 µg/ml leupeptin. Then the radioactivity in 100 µl of solubilized sample was counted in a liquid scintillation counter. After addition of 5 µl anti-bovine albumin antiserum to 500 µl of solubilized sample and incubation at 4°C overnight, the antigen-antibody complexes were bound to 7 mg (dry mass) of protein-A-Septarose 4B, washed four times with solution A and
twice with 50 mM sodium phosphate buffer, pH 7.5, and eluted by incubation with 20 μl of 0.1 M Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, 5% SDS and 10% glycerol at 95°C for 5 min. The 20 μl of eluted protein samples were analyzed by 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) [12] and subjected to fluorography using ENLIGHTNNG (DuPont, Boston, MA, U. S. A.) according to the instruction manual of the company.

Glycogenolysis: Glycogenolytic activity of hepatocytes was assessed from the epinephrine-induced release of glucose from the cells. The freshly isolated or cultured hepatocytes were washed twice with Hanks' solution not containing glucose (0.14 M NaCl, 5.4 mM KCl, 0.33 mM Na₂HPO₄, 4 mM NaHCO₃, 1.2 mM CaCl₂, 0.4 mM KH₂PO₄, 0.4 mM MgSO₄), and incubated at 37°C for 30 min at various concentrations of epinephrine. Glucose released into the solution was assayed by the glucose oxidase method (Glucose B-test Wako, Wako, Tokyo). The protein content of hepatocytes was determined by the method of Lowry et al. [13].

RESULTS

The efficiency of the isolation of bovine hepatocytes was shown in Table 1. Cell yield and total cell number were 0.1–3.6 x 10⁷ cells/g liver and 2–33 x 10⁸ cells, respectively. Cell viability was 70–92%, as assessed by trypan blue exclusion test.

Microscopic observations revealed that hepatocytes attached to the plastic plates and began to spread after 3 hr in culture and formed monolayers after 24 hr, which were retained for up to 6 days (Fig. 2). Both isolated and cultured bovine hepatocytes are microscopically homogeneous and exhibited characteristic shapes similar to those of hepatocytes from rats and some other animal species [2, 18].

To assess protein synthesis and secretion during culture, we measured the radioactivity of TCA-insoluble materials in the medium which were metabolically labeled with [³⁵S]-methionine. As shown in Fig. 3, ³⁵S-incorporation

![Graph showing ³⁵S incorporation into TCA-insoluble fraction over days in culture.](image)

Fig. 3. Synthesis and secretion of total proteins during culture. Bovine hepatocytes were cultured for 2 hr in the presence of [³⁵S]-methionine, and the incorporation of ³⁵S into TCA-insoluble fractions in the medium was measured. Each point is the mean of values in at least 2 experiments.

**Fig. 2.** Phase contrast microscopy of freshly isolated and cultured bovine hepatocytes. A-D were cultures for 0, 3, 24 and 72 hr, respectively. Bar = 100 μm.
was low in freshly isolated hepatocytes, but increased much after 1 day of culture and decreased gradually during 6 days.

Then, synthesis and secretion of albumin, one of the liver-specific metabolic activities, were examined by immunoprecipitation of $^{35}$S-labelled proteins by an antiserum against bovine albumin. SDS-PAGE analysis gave a band of 67 kDa, which was equal to the molecular mass of albumin (Fig. 4). The albumin band was weak in freshly isolated hepatocytes, but became dense after 1–3 days in culture. After 6 days in culture, the albumin band returned to be weak again.

The glycogenolytic response to epinephrine was examined by measuring glucose release into the medium. As shown in Fig. 5, epinephrine stimulated glucose release in a dose-dependent manner in freshly isolated hepatocytes. However, the response to epinephrine was several times greater when hepatocytes were cultured for 1–3 days. After 6 days in culture the response returned nearly to that of isolated hepatocytes.

**DISCUSSION**

In this study, we demonstrated that hepatocytes isolated from the caudate lobe of bovine liver formed monolayers in culture for up to 6 days, maintaining the activities of albumin synthesis and epinephrine-induced glycojenolysis, both of which are typical liver-specific functions.

To perform primary culture successfully, it is important to isolate hepatocytes with high yield and viability. In preliminary studies, we tried to isolate hepatocytes by shaking cut liver pieces in a collagenase solution, but this method was insufficient to digest connective tissue, and resulted in low cell yield and viability. In contrast, successful digestion could be performed by perfusing the caudate lobe with EGTA and collagenase. The viability, yield and total cell number of hepatocytes in this method were comparable to those reported for rat hepatocytes [5, 9, 18]. It should be noted that our perfusion apparatus is simple and inexpensive compared with those of others [8, 17], and thereby can be used more easily.

In preparation of primary culture of bovine hepatocytes, the method generally used for rat hepatocytes was applied. Bovine hepatocytes formed monolayers in primary culture on plastic plates coated with rat tail collagen. At the onset of culture, behavior of spreading onto plates was similar to that of cells from other species [2, 18]. Although Van’t Klooster *et al.* [19] reported that collagen coat is not necessary for primary culture of bovine hepatocytes, we preliminary observed lowered efficiency of cell attachment on plates without collagen coat. The difference may be due to the choice of plastic plates.

It is established that rat hepatocytes lose many cell-specific activities immediately after isolation, but recover them during culture [5, 9, 18]. However, previous studies on bovine hepatocytes indicated a rapid decrease in drug-metabolizing enzyme activity, which was an only function tested in these studies [17, 19]. Therefore, two other representative functions of liver, albumin synthesis and glycogenolytic response, were investigated in this study. Albumin is known to be synthesized exclusively and

![Fig. 4](image)

**Fig. 4.** Synthesis and secretion of albumin during culture. The TCA-precipitable materials labeled with $^{35}$S-methionine obtained from each 300 μl medium were solubilized and the half part of the solubilized sample was immunoprecipitated with an antiserum against bovine albumin, subjected to SDS-PAGE and fluorography as described in "MATERIALS AND METHODS". Same amount of the medium was processed after 0 (Lanes 1 and 2), 1 (Lanes 3 and 4), 3 (Lanes 5 and 6), and 6 (Lanes 7 and 8) days in culture.

![Fig. 5](image)

**Fig. 5.** Epinephrine-stimulated glycogenolysis in freshly isolated and cultured bovine hepatocytes. Freshly isolated and cultured hepatocytes were incubated for 30 min with epinephrine at the concentrations indicated. Basal glucose releases in the absence of epinephrine after 0 (●), 1 (○), 3 (■), and 6 (□) days in culture were 43.8, 84.4, 76.4, and 32.2 μg glucose/hr/mg cellular proteins, respectively. Each point is the mean of values in at least 2 experiments.
abundantly by hepatocytes [6, 10], and thereby a suitable marker for hepatocytes maintaining liver-specific functions. Synthesis and secretion of albumin, as well as whole proteins, were much enhanced by culturing bovine hepatocytes. Unfortunately, albumin synthesis decreased after 6 days in culture. A similar result was also reported for rat hepatocytes [5, 9, 18]. The reason for this decrease in albumin synthesis is not clear at present. Because the pattern of the changes in albumin and whole protein secretion is almost parallel, some general mechanism(s), not specific to albumin, may be involved for this decrease. Similarly to protein synthesis, glycogenolytic response to epinephrine was higher in cultured hepatocytes. This result accords favorably with the results of rat hepatocytes [5, 9], and suggests that glycogenolytic response was improved by culturing bovine hepatocytes. Epinephrine-induced glycogenolysis is mediated by the signal transduction pathways from adrenergic receptors to an activation of glycogen phosphorylase through the cascades involving trimeric GTP-binding protein, cyclic AMP, calcium ion, and phosphatidylinositol. These pathways seem to work well in the present culture system.

Thus, primary cultured bovine hepatocytes maintained some biochemical functions for several days in culture. These results are a contrast to those of Shull et al. [17] and Van’t Klooster et al. [19], who reported a rapid decrease in drug-metabolizing capacity of bovine hepatocytes in culture. It should be stressed, however, that while a similar loss of drug-metabolizing activity was often observed in rat hepatocytes, the rat culture has been recognized as a useful model for other biochemical studies [5, 18]. Moreover, although there are some human and rat cell lines that retain hepatocyte-specific functions [11, 15], such cell lines from ruminant species have not been reported so far. Thus, primary cultured bovine hepatocytes could be useful for studies on ruminant liver functions.

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