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Characterization of human hepatocytes isolated from non-transplantable livers

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B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction: The successful use of hepatocytes depends on a reliable demonstration of the functional and morphological integrity of isolated cells. Herein we investigated whether the isolation and cryopreservation of primary human hepatocytes can compromise cell viability and liver-specific characteristics.

Materials and Methods: Hepatocytes were isolated from encapsulated human liver segments by a modified 2-step perfusion technique. Isolated cells were Percoll-purified, cryopreserved, and stored in liquid nitrogen for 1–12 months. For rapid assessment of fresh and cryopreserve/thawed hepatocyte yield and viability, the cells were stained with trypan blue or labeled with fluorochromes. For immunocytochemical analysis, the cells were labeled with monoclonal antibodies for the presence of the following antigens and chemokines: CD3, CD45Ro, CD45Ra, CD34, CD68, CD90, CD95, CD20, HLA-DR, Ki67, PCNA, Bcl-2, p53, CXCR3, CXCR4, and SDF-1. The cells were tested for several specific functions, such as ureagenesis, energy status, MTT activity, lactate dehydrogenase leakage, and total CYP450 content.

Results: Assessment of both freshly isolated (Percoll-purified) and cryopreserved/thawed hepatocytes revealed a low constitutive level of contamination by non-parenchymal cells compared with crude (unpurified) preparations and tissue sections. All viable hepatocytes showed intact morphology and retained CYP450 protein, energy status, and urea synthesis.

Conclusion: Modifications in hepatocyte preparations, such as depletion of dead, damaged, and non-parenchymal cells, improves cell purity, which can be adapted to further evaluation of hepatocyte immunogenicity. These data illustrate the importance and feasibility of human hepatocyte banking.

Key words: **hepatocyte isolation • human • cryopreservation • non-parenchymal cells**

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INTRODUCTION

Classically, liver cells are divided into parenchymal cells (hepatocytes) and non-parenchymal cells. Hepatocytes represent 70–75% of all cells in the liver and perform the majority of liver-related functions, including metabolic transformation of xenobiotics, clearance of toxins, disposal of bile pigments, and synthesis of cholesterol, fatty acids, and plasma proteins (albumin, blood coagulation proteins, complement, protease inhibitors). The remaining 25–30% are non-parenchymal cells and comprise unique liver sinusoidal epithelial cells), cells with accessory immune function (Kupffer, NK, NK.T, stellate, and biliary epithelial cells), and intrahepatic immigrant T and B lymphocytes.

Data from reported experiments indicate that properly isolated and prepared human hepatocytes retain their liver-specific characteristics and can provide a valuable *in vitro* liver model in both research and clinical applications[1, 9, 14, 33]. There is a pressing need for alternative therapeutic methods effective in the management of patients with inherited and acquired liver insufficiency. Therefore, improvements in liver cell isolation have generated enthusiasm for allogeneic hepatocyte transplantation as a potential treatment to provide temporary liver function. Recent clinical studies suggest that hepatocyte transplantation may be useful in bridging patients to whole-organ transplantation and in certain metabolic liver diseases[4, 10, 11, 19, 41, 44]. However, the successful use of hepatocytes in clinical trials depends on a reliable demonstration of the functional and morphological integrity of isolated cells. Pre-clinical evaluation of hepatocytes involves microscopic examination and precise *in vitro* analysis of cell viability and multiple metabolic activities to identify the capacity of these cells to remain functional *in vivo* after transplantation. Most experimental and clinical studies in human cell transplantation have used adult primary hepatocytes. To study liver functions *in vitro,* or for the clinical use of cell transplantation and bioartificial liver devices, a constant supply of primary human hepatocytes has to be provided. Due to the limited availability of these valuable cells, the development of effective isolation systems that allow long-term storage and subsequent cell cultivation and/or their use in clinical applications is of great importance. Although successful isolation and transplantation of animal hepatocytes has been demonstrated[35, 46], there are no reports describing the investigation of isolated human hepatocytes from the Polish perspective. Our specific goal was to investigate whether the isolation and banking of primary human hepatocytes can compromise cellular integrity, which may influence their potential use in clinical applications.

MATERIALS AND METHODS

Chemicals

Media and reagents used for hepatocyte isolation, including Hank's balanced salt solution (HBSS), Leibovitz (L-15), phosphate-buffered saline (PBS), ethyleneglycol-bis(aminoethylether)-tetraacetic acid (EGTA), glucose, penicillin/streptomycin, media supplements, fetal calf serum (FCS), dimethyl sulfoxide (DMSO), and collagenase (type I) were purchased from Sigma Chem. Corp. (USA). University of Wisconsin solution was obtained from DuPont Pharmaceuticals, Percoll was from Pharmacia Biotech, and the fluorescent dyes 5,6-carboxyfluorescein diacetate succinimidyl ester (CFDA) and Calcein-AM were from Promega. Becton Dickinson (USA) supplied all antibodies for FACS analysis. Monoclonal antibodies and all other reagents for immunocytochemical analysis were from Dako (Denmark). The lactate dehydrogenase (LDH) determination kit, reagent system for urea nitrogen, and MTT (3-(4, 5--dimethylthiazol-2-yl)-2, 5-diphenyl tetrasolium bromide) kit were from Sigma Chem. Corp. (USA).

Hepatocyte isolation

Non-diseased human livers were obtained from the Department of Vascular Surgery, Medical Academy of Wrocław, Poland. All livers used for hepatocyte isolation were collected from brain-dead, heart-beating

Table 1. Demographics and clinical characteristics of liver donors

Donor no.	Age	Sex/race	AST U/l	ALT U/l	Bilirubin mg (%)	Fat content (%)	Cause of death	Reason not transplanted
1	20	M/C	108	66	0.8	55 (micro)	closed head	fat content
2	52	M/C	30	23	0.5	25 (micro)	intracranial	medical/social history
3	1.5	F/C	159	403	0.8	0	asphyxia	no donor – recipient mat
4	54	M/C	63	22	0.5	15 (micro) 35 (macro)	closed head	fat content alcoholism
5	24	M/C	28	38	0.4	35 (micro)	cerebral aneurysm	fat content

All livers were collected from brain-dead, heart-beating donors and at the time of referral, all specimens had been deemed unsuitable for transplantation. M – male, F – female, C – Caucasian, micro – microvesicular steatosis, macro – macrovesicular steatosis.

donors and were approved by the donor's families for research use. Donor demographic data and clinical parameters are summarized in Table 1. The livers were protected from ischemic injury by flushing with ice-cold University of Wisconsin solution immediately after vascular clamping and re-section, placed on ice, and transported immediately to our laboratory. The interval from initial flush to hepatocyte isolation (cold ischemic time) was 12–24 h. At the time of referral, all specimens had been deemed unsuitable for transplantation for one or more reasons (e.g. high fat content, donor medical/social history, biochemical profile, no donor-recipient match) and all protocols were approved by the Bioethics Committee and in accordance with the Institutional Review Board of the Medical Academy (Wrocław, Poland). Hepatocytes were isolated from the encapsulated human liver segments (preferably the left lateral segment) by a modified 2-step perfusion technique as described previously[36]. Briefly, the livers were flushed under a sterile biosafety hood through the portal vein and/or hepatic vessels (re-circulation technique) with 3 l of calcium--free HBSS pre-warmed to 37°C supplemented with 0.5 mM EGTA, and then with 0.05% collagenase solution until the tissue was fully digested. The digestion time for each preparation was in the range of 35–47 min. The digested liver was removed, immediately cooled with ice-cold L-15 medium, and the final cell suspensions were centrifuged twice (65×g for 3 min at 4°C), and the medium aspirated. The crude hepatocyte suspension was purified on a Percoll gradient (at optimal concentrations of Percoll in the range of 29–31%).

Hepatocyte cryopreservation

Freshly isolated hepatocytes were cryopreserved according to a procedure described previously[28]. Briefly, Percoll-purified hepatocytes were suspended at a density of 7×10^6/ml of cryopreservation mixture (70% University of Wisconsin solution, 20% FBS, 10% DMSO) and aliquoted into 2-ml Nalgene cryovials. The vials were placed into Nalgene freezing buckets and left in a freezer at –80°C for 5 h and then transferred into liquid nitrogen (LN_2), where they were stored for 1–12 months. For cell thawing, the vials were removed from the LN_2 and immediately thawed in a 37°C water bath, suspended in L-15 medium supplemented with 10% FCS, centrifuged twice (65×g for 3 min at 4°C), and then processed as fresh preparations.

Hepatocyte culture

The final cell suspension was centrifuged and the medium aspirated. The cells were resuspended in L-15 medium supplemented with 10% FCS and 100 units of penicillin and streptomycin, dispensed into 6-well (1.0×10^6/well) collagen-coated plates, and incubated at 37°C in a humidified atmosphere of 95% air 5% CO_2. The culture medium was changed initially after 3 h of incubation and then every 24 h, using a serum-free William's E medium containing 0.1 μM dexamethasone, 100 ng/ml insulin, 1 μg/ml glucagon, 10 μg/ml transferrin, 3 nM selenium, 100 units of penicillin and streptomycin, and 20 ng/ml of HGF (for cryopreserved/thawed cells only).

Determination of hepatocyte viability

The yield and viability of freshly isolated or cryopreserved hepatocytes were estimated by trypan blue staining. For another rapid assessment of hepatocyte viability, the cells were also labeled with fluorochromes. Three million cells were suspended in PBS medium enriched with 10% FCS and fluorescein dye CFDA was added from a stock solution to a final concentration of 5 μM/l. The cultured cells were washed twice with serum-free L-15 medium and then incubated for 15 min with CFDA or Calcein-AM, after which the medium was changed to L-15 supplemented with 10% FCS. The fluorescence intensity was determined using a fluorescent microscope (Axioplan 2). Culture plates were viewed by phase-contrast microscopy (Nikon TMS).

Immunocytochemistry and flow cytometry

For immunocytochemical and histochemical analysis, both tissue sections and isolated hepatocytes were prepared. Liver tissue fragments were snap-frozen and cryostat sectioned according to standard procedures using a Tissue Tek II Cryostat. Alternatively, 10^5 unfixed isolated hepatocytes were suspended in L-15 medium supplemented with 5% FCS, spun for 5 min in Shandon (Southern) Cytospin, and then stained. All specimens were labeled with monoclonal antibodies for the presence of the following antigens: CD3, CD45Ro, CD45Ra, CD34, CD68, CD90, CD95, CD20, HLA--DR, Ki 67, proliferating cell nuclear antigen (PCNA), Bcl-2, p53, and cytokeratin. All antibodies were diluted 1:30–1:500 or prepared according to the manufacturer's instructions. To evaluate the expressions of chemokines and chemokine receptors, all specimens were stained with distinct anti-human CXCR3, CXCR4, and SDF-1 monoclonal antibodies. Briefly, all slides to be probed were air-dried and fixed in methanol-acetic acid for 10 min. After incubation for 5 min in 0.05 M Tris buffer, they were treated with monoclonal antibodies, incubated for 30 min at room temperature, and then rinsed twice in Tris buffer. Secondary anti-mouse antibody conjugated with alkaline phosphatase was added and, after 30 min of incubation, all the slides were rinsed in Tris buffer. After double rinsing in Tris buffer, all the slides were incubated for 8 min with the Fast-Red

Substrate System (0.6 mg/ml in Tris buffer), rinsed, and then counter-stained in hematoxylin. The specimens were examined by a DOCUVAL (Carl Zeiss) light microscope. For FACS analysis, the cells were washed twice in PBS buffer (without Ca^{2+}, Mg^{2+}) and then incubated with FITC alone or with fluorescein conjugated anti-class I or anti-class II monoclonal antibodies. Flow cytometry was performed using a FACS Calibur (Becton Dickinson, USA).

Hepatocyte function assays

The energy status of hepatocytes cultured overnight was evaluated by measuring intracellular ATP content based on a bioluminescence assay using a multilabel plate reader (Perkin Elmer Wallac, Victor 1420). To quantify total cytochrome P450 (CYP450) content in isolated hepatocytes, the cells were homogenized and centrifuged at 2000×g for 3 min. The resulting supernatants were centrifuged at 9000×g for 20 min. The pellet obtained was resuspended in PBS and centrifuged at 105,000×g for 90 min. The final microsomal pellet was resuspended in PBS at a concentration of 4 mg protein/ml. To measure the total CYP450 protein content, the differential spectrum was recorded according to the method of Matsubara et al.[28] To measure ureagenesis, 1×10^6 cells were suspended in serum-free L-15 medium and incubated with addition of 5 mM ammonium chloride for 0, 1, 2, and 4 h. After incubation, the cells were centrifuged for 5 min and the urea concentration in the supernatant was measured using a spectrophotometric test kit. LDH release from freshly isolated hepatocytes was estimated using a diagnostic Lactate Dehydrogenase kit. The absorbance was measured at 340 nm using a Beckman spectrophotometer. The activity of mitochondrial dehydrogenases was estimated using the MTT assay. Briefly, the cells were incubated with MTT reagent in 96-well plates for 2 h ($0.2–1\times10^6$ cells/well) and then centrifuged for 5 min at 200×g. The supernatant was decanted, and 100 µl of PBS was added to each well and centrifuged again. The cell pellets were treated with 100 µl of acidified alcohol and incubated for 2 h at room temperature. The absorbance was measured at 570 nm on a plate reader.

Statistical analysis

Data were expressed as means ±SE and evaluated by Student's *t*-test for comparison between fresh and cryopreserved hepatocytes isolated from the same liver. A p value <0.05 was considered statistically significant.

RESULTS

Hepatocyte yields after collagenase dissociation (before Percoll) averaged 3.2×10^7 cells per gram of wet tissue. The average final hepatocyte viability was 72% for the crude preparations and 89% after Percoll purification. Examination of cells labeled with CFDA (suspensions) or Calcein-AM (cell monolayers) revealed an even distribution of fluorescence within the viable hepatocytes (Fig. 1 and 2). Viable hepatocytes showed typical and intact morphology as demonstrated by light microscopy. In the suspensions, most of the cells were binucleated and spherical in shape. The appearance of cryopreserved/thawed hepatocytes was similar to that of freshly isolated, intact cells. Freshly isolated hepatocytes attached to collagen pre-coated dishes within 4 h after plating and formed confluent monolayers by 12 h, at which point the cells had spread, flattened, and formed a monolayer across the dish (Fig. 3). In con-

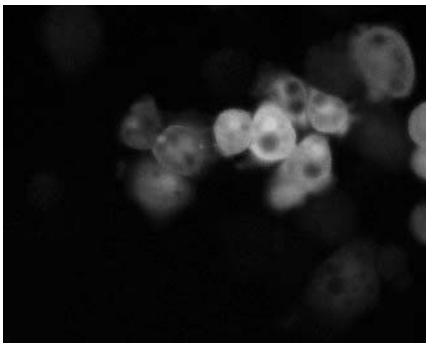

Figure 1. Fluorescent photomicrograph of freshly isolated (Percoll-purified) human hepatocytes in suspension, stained with CFDA.

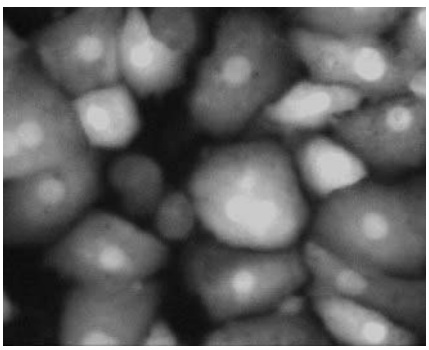

Figure 2. Fluorescent photomicrograph of cultured for 24-h human hepatocytes (fresh, Percoll-purified), stained with Calcein-AM.

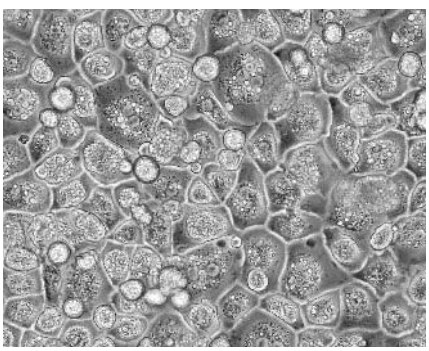

Figure 3. Phase-contrast photomicrograph of freshly isolated, Percoll-purified human hepatocytes in 12-h monolayer culture.

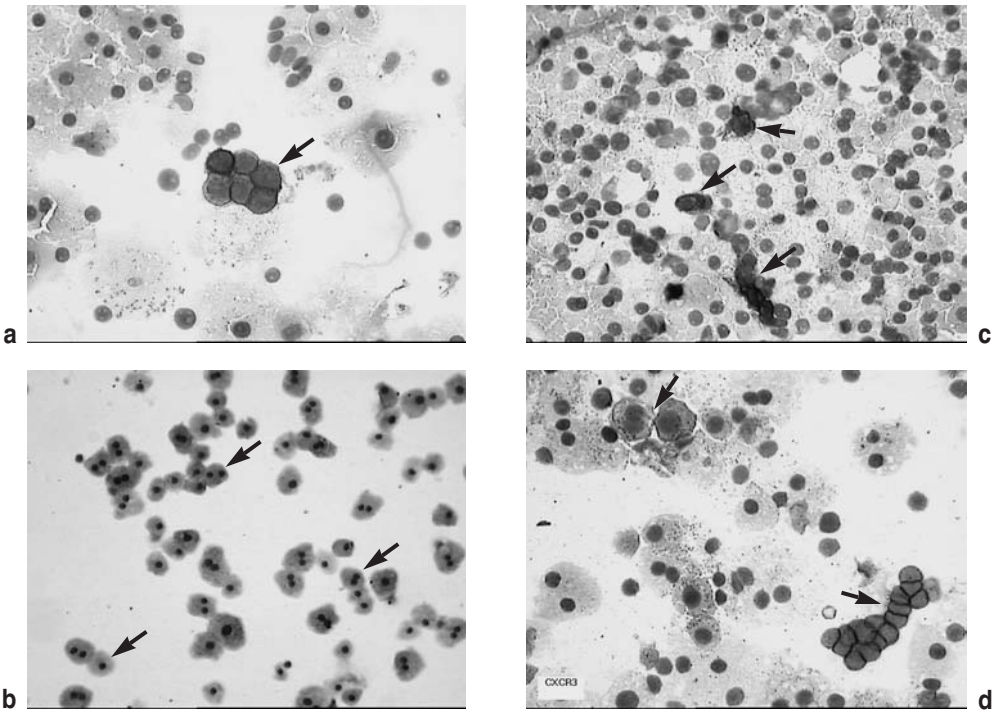

Figure 4. Cell markers tested in cytospin preparations of Percoll-purified hepatocytes: **a** – $CD90^+$ cells (donor no. 2), **b** – $CXCR4^+$ cells (donor no. 2), **c** – $CD34^+$ cells (donor no. 1), **d** – $CXCR3^+$ cells (donor no. 1).

trast, the plating efficiency of cryopreserved hepatocytes, plated immediately after thawing, ranged 35–40%. Attachment of thawed and then Percoll-purified cells was higher and ranged 65–85%.

Staining with the cell markers in all the tested tissue sections showed the appearance of scattered and, in part, clustered $CD3^+$, $CD45Ra^+$, $CD45Ro^+$, $CD68^+$, DR^+, and $CD90^+$ non-parenchymal cells, mostly located in portal triads. $CD34^+$ cells poor in cytoplasm were also observed close to the portal triads. In some focal areas, cells showing the CD20 surface antigen were observed in 1 out of 5 cases. Immunocytochemical staining for the proliferation marker Ki67 revealed slight reactivity in all the studied slides. However, there were no indications for the immunoreactivity of PCNA, and faint expression of p53 was observed only in one tissue section. Bcl-2--related apoptotic proteins were detected in 2 cases, and CD95 immunoreactivity was not observed. Although the light immunohistochemical intensity of CXCR3/CXCR4 was variable and comparable in all tested cases, most of the lymphocytes expressed CXCR3 and only some stained weakly for CXCR4. Crude preparations of isolated cells (freshly isolated, before Percoll) had similar expressions of the markers assayed for chemokines.

Assessment of Percoll-purified and cryopreserved/ /thawed hepatocytes revealed a low constitutive level of contamination by non-parenchymal cells compared with crude fresh preparations (Fig. 4 a, c; Fig. 5 a, b, e, f). Three of the tested antibodies (monoclonal anti-CD68, DR, and CD90) provided reliable immunoreactivity in hepatocyte cytospin specimens. Also, there was a positive reaction in 3 out of 5 cases after staining for CD34. Apoptotic protein Bcl-2 was visible in 1 out of 5 cases. Immunohistochemical staining with the chemokine markers showed the appearance of weak expressions of CXCR3 and CXCR4 in two cases (Fig. 4 b, d). The data on antigenic and chemokine expression levels in the panel of human liver tissue sections and isolated hepatocytes are summarized in Table 2.

FACS analysis of HLA class I and class II (DR) antigens on Percoll-purified hepatocytes (recovered from cryopreservation) revealed that 98%, and 95% of cells in analyzed preparations, were positive for HLA class I antigens (Fig. 6). Four out of 5 tested preparations revealed a relatively moderate level of cytokeratin expression (Fig. 5 c, d).

Both freshly isolated and cryopreserved hepatocytes retained CYP450 protein. The levels of total CYP450 protein in 3 hepatocyte samples analyzed (after 24-h culture) showed a very consistent pattern and averaged 0.270 nmol/mg protein for freshly isolated cells and 0.279 nmol/mg protein for cryopreserved cells. Energy status, evaluated by the intracellular ATP concentra-

Table 2. Phenotype antigens in liver tissue sections and hepatocyte preparations

Antigens	Donor number				
	1	2	3	4	5
Non-parenchymal cells (TC/PP)					
CD3	+/–	+/+	+/–	+/–	+/–
CD45 Ro	+/–	–/nt	–/nt	–/nt	–/nt
CD45 Ra	+/–	+/+	–/–	+/–	+/–
CD68	++/++	++/+	++/+	++/–	+++/++
DR	+++/+	+/+	++/+	+++/+	+++/+
Cytokeratin – CK (TC/PP)					
CK	–/++	–/+	–/+	–/–	–/+
Lymphocytes, stem cells, chemokines (TC/PP)					
CD20	–/–	–/–	–/–	–/–	+/–
CD90	++/+	++/+	+++/+	++/–	+++/+
CD34	–/+	–/+	–/–	–/–	++/+
CXCR3	+/+	+/–	+/–	+/–	–/–
CXCR4	+/–	–/+	+/–	–/–	–/–
Proliferation antigens (TC/PP)					
PCNA	nt	–/nt	–/nt	–/–	–/–
Ki67	++/–	+/nt	++/–	+/–	–/–
Apoptosis antigens (TC/PP)					
CD95	–/–	–/–	–/–	–/–	–/–
p53	–/nt	–/nt	–/nt	–/–	+/–
Bcl-2	nt/+	++/–	–/–	+/–	–/–

All specimens were labeled with monoclonal antibodies for the presence of the tested antigens, chemokines, and cytokeratin. TC – tissue sections and crude (before Percoll) hepatocyte preparations, PP – Percoll-purified hepatocytes recovered from cryopreservation, nt – not tested.

tion measurements, was also maintained in all tested specimens (Fig. 7). The ATP content in fresh hepatocytes averaged 1.20±0.96 nmol/10^6 cells. Significant depletion of intracellular ATP was observed in hepatocytes after cryopreservation. The MTT assay demonstrated high colorimetric activity of mitochondrial dehydrogenases in all tested preparations; however, in the current studies the reaction product was expressed more intensely in cryopreserved cells than in fresh preparations (Fig. 8). Activity of LDH released into the supernatants by freshly isolated hepatocytes incubated for 3 h averaged 842.8±18.2 U/10^6 cells; the small difference in mean activities between fresh and cryopreserved preparations was not significant. Both freshly isolated and cryopreserved/thawed hepatocytes synthesized and secreted urea into the medium during the 4-h incubation period. The average rate of urea synthesis in fresh preparations was 180.5±12.3 nmol/10^6 cells. No significant difference in urea synthesis was observed between fresh and cryopreserved cells in 5 studied livers.

DISCUSSION

In recent years, many reports have confirmed that properly isolated human hepatocytes retain their metabolic activity and cellular morphology, and they are the most common cellular model in current studies of drug metabolism and engineered therapies[31, 32]. In the clinic, human hepatocytes are more appropriate than animal hepatocytes in terms of immunological reaction. The outcomes of all clinical trials in this field were evaluated as a promising bridging mechanism for maintaining life prior to solid-organ transplantation. However, one of the major problems limiting the application of human hepatocytes is the chronic shortage of liver donors. Organs that were considered unsuitable for liver transplantation in earlier years are more routinely used as the demand for transplantation increases[38]. Although the successful isolation of highly viable human hepatocytes from livers rejected for whole-organ transplantation remains a significant challenge for many laboratories, each attempt of this procedure brings us closer to improving the existing technologies in this field. Thus the establishment of techniques for the large-scale isolation, culture, and characterization of hepatocytes from unused human livers is of great importance. Moreover, the ability to preserve and store these valuable cells would greatly facilitate biomedical research. The successful isolation and cryopreservation of hepatocytes adds great potential to both clin-

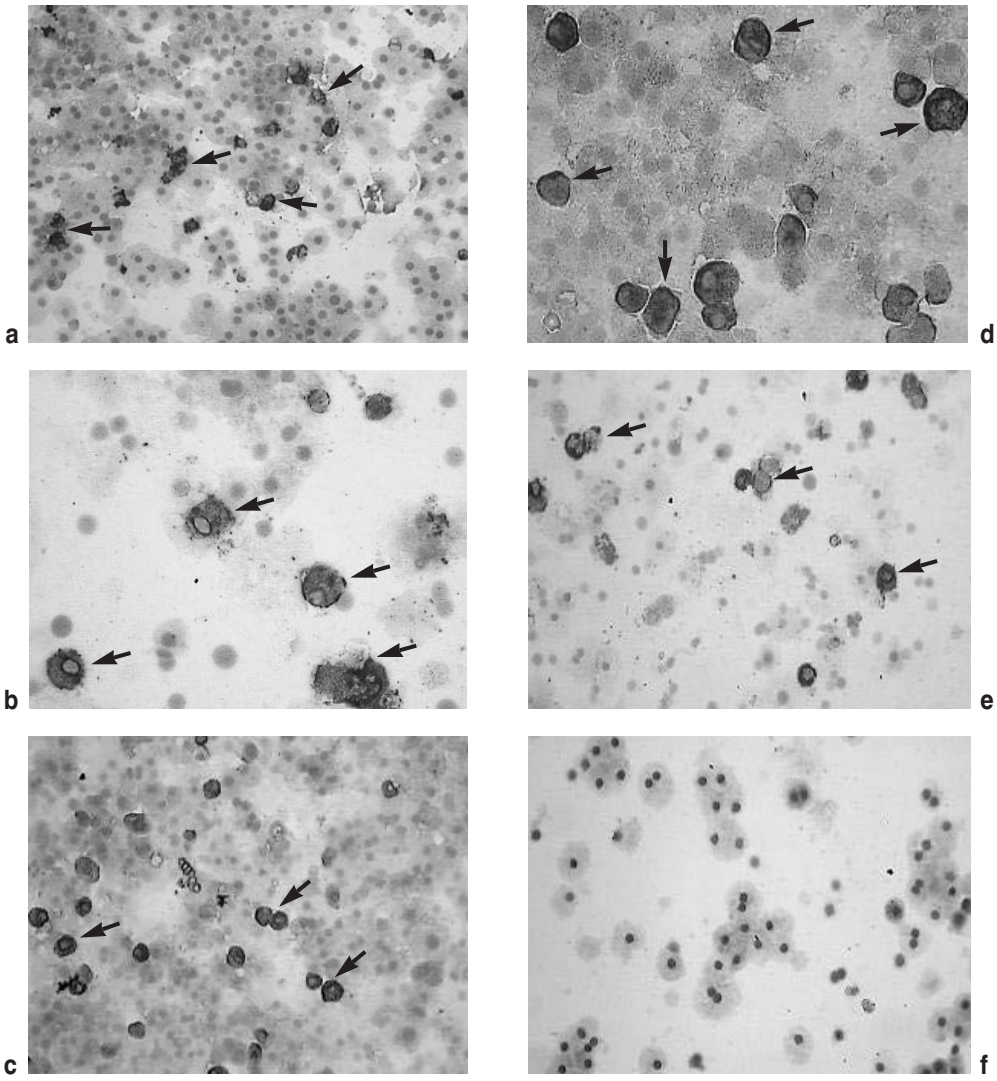

Figure 5. Isolated hepatocytes in cytospin preparations: **a**, **b** – $CD68^+$ cells in purified preparations (donor no. 5), **c**, **d** – cytokeratin-positive, Percoll-purified hepatocytes (donor no. 5), **e** – crude preparations (before Percoll) with scattered DR^+ cells (donor no. 5), **f** – Percoll-purified preparations with no observed contamination by antigen-presenting cells (donor no. 5).

ical and research programs; however, an efficient and reproducible hepatocyte production and preservation method is enormously difficult to establish. Parenchymal liver cells can be considered as extremely fragile cellular components and require very specific microenvironmental conditions to maintain the cell phenotype *in vitro*.

In our experiments, the quantity and viability of freshly isolated (or cryopreserved/thawed) hepatocytes were estimated by trypan blue exclusion or labeling with CFDA and Calcein-AM according to standard procedures described for isolated hepatocytes. The fluorescent dyes, originally used in lymphocyte migration studies, now permit a clear identification of hepatocytes by fluorescent microscopy. Non-fluorescent CFDA is taken up by diffusion and converted by cytoplasmic esterases to carboxyfluorescein, which is retained by viable cells. Calcein AM is hydrolyzed by endogenous esterase into the highly negatively charged green-fluorescent calcein. These probes measure two recognized parameters of cell viability: plasma membrane integrity and intracellular esterase activity, which can also be considered as an index of cellular metabolic rate. Thus, staining with fluorochromes is a method that correlates closely with hepatocyte function and allows for the simultaneous quantitative determination of live and dead cells[36]. Compared with trypan blue exclusion, this method is easier to read, more stable, and has fewer staining artifacts, which is consistent with findings previously reported[3, 29].

Based on our laboratory experience (unpublished), the viability (before Percoll) of fresh hepatocytes isolated from high-quality human liver samples obtained

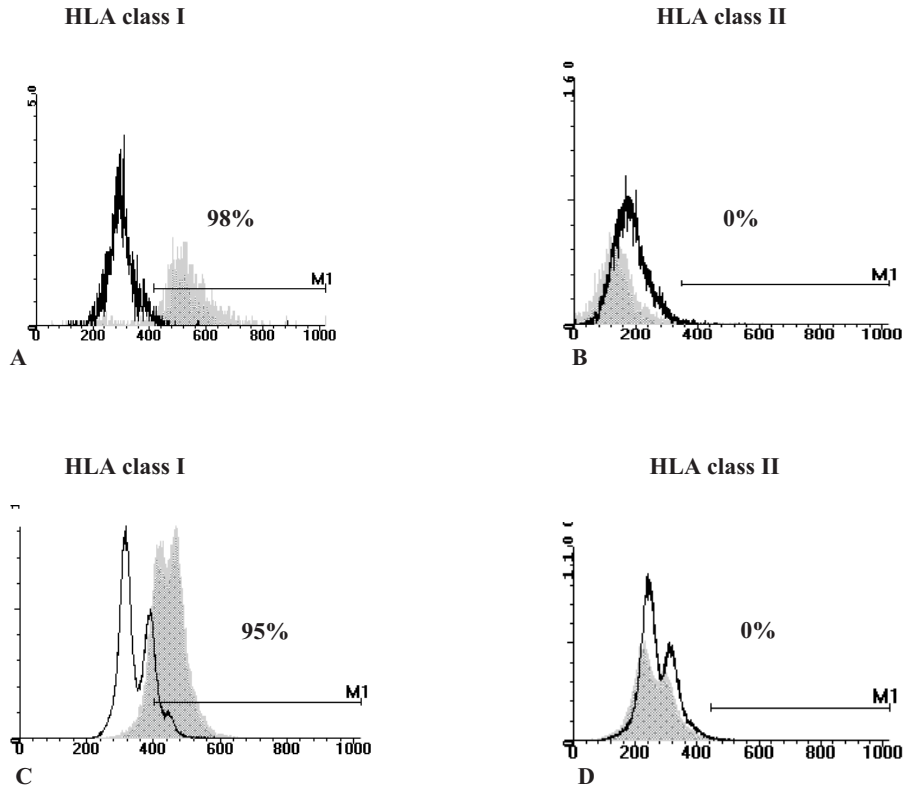

Figure 6. FACS analysis of HLA class I and class II antigens in representative Percoll-purified hepatocyte preparations (recovered from cryopreservation): **A**, **C** – cells labeled with FITC-conjugated anti-class I antibodies (shadowed area); **B**, **D** – low contamination of isolated hepatocytes by antigen-presenting cells labeled with FITC-conjugated anti-class II antibodies (shadowed area).

in the course of liver surgery should be around 85–95%. However, uncertainty exists with respect to the quality of hepatocytes processed from donor livers that were considered unsuitable for liver transplantation. Because of the critical shortage of donor livers for patients, the number of these organs that might be available for cell isolation has recently decreased dramatically. Hence the livers available for hepatocyte transplantation are, in most cases, severely damaged or contain substantial fatty, fibrotic, or cirrhotic changes; these factors significantly influence the overall outcome of cell isolation procedures. Considering the difficulties in human liver acquisition, and the poor quality of processed organs, obtaining a sufficient number of cells with a high degree of viability is extremely difficult. Therefore the focus of the standard hepatocyte purification method is the elimination of all potential contaminants, including dead and damaged cells and cell debris. For this reason, all initial preparations of the freshly isolated human hepatocytes in this study were further purified by centrifugation in Percoll gradient, which is a well-established method in many laboratories[1, 20, 26, 27]. However, treatment of hepatocyte preparations with Percoll causes, at the same time, a significant depletion of non-parenchymal cells, including Kupffer cells, stellate and endothelial cells, and lymphocytes. The rationale for eliminating dead

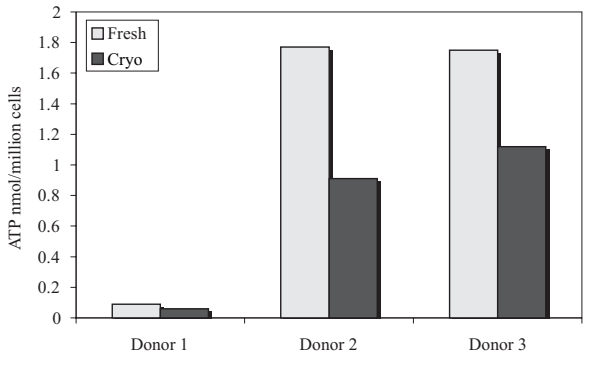

Figure 7. ATP content in freshly isolated (Percoll-purified) and cryopreserved/thawed hepatocytes (immediately after thawing) hepatocytes (nmol/10^6 cells); $p<0.05$ vs. fresh hepatocytes.

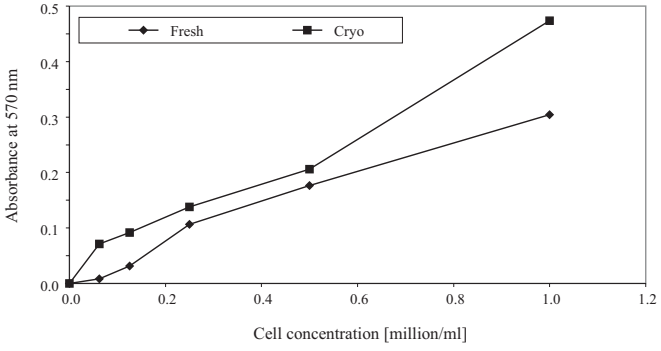

Figure 8. MTT activity in freshly isolated (Percoll-purified) and cryopreserved hepatocytes; the number of viable hepatocytes is proportional to the concentration of the MTT reaction product, as determined by the optical density.

or damaged cells is self-evident, whereas for some applications the retention of viable non-parenchymal cells may be desirable. According to several reports, co-culture of hepatocytes with non-parenchymal cells may have a beneficial effect in artificial, 3-dimensional network configuration[15, 24]. Extension of this procedure to the development of a bioartificial liver support is well established and believed to play an essential role in maintaining cell-cell interactions, both homotypic and heterotypic, which greatly augments liver-specific function. However, there is substantial evidence that Percoll-purified isolated hepatocytes can also survive in standard monolayer cultures, maintained for a number of days, which depends on the culture models. Thus, human pure hepatocytes represent a well-accepted *in vitro* cell culture system for studies of drug metabolism, enzyme induction, transplantation, and various pharmacological and toxicological investigations[17, 20, 39, 40, 45]. To provide information on the quality of isolated hepatocytes, it is highly recommended to evaluate the plating efficiency of these cells in primary cultures. Freshly isolated human hepatocytes in culture have been reported to attach well to collagen-coated plates, which is consistent with our observations. Modifications in the culture media, including supplementation with hormones, growth factors, minerals, and vitamins, can stabilize hepatocyte morphology, and activity to 8–14 days. However, there is a dramatic decline in hepatocyte plating efficiency in most cryopreserved/thawed preparations. Several studies have shown that cryopreservation may trigger apoptosis in isolated animal hepatocytes, which could be critical for the ability of the cells to attach and form a monolayer[12]. Nevertheless, under appropriate thawing and culture conditions, hepatocytes recovered from cryopreservation attach well to plates coated with collagen and remain viable for extended periods of time[13, 14, 40].

Reports on the survival and function of isolated hepatocytes, as well as the importance of donor non-parenchymal cells *in vivo*, are controversial. Studies have highlighted that allogeneic hepatocytes in a murine model stimulate a strong cell-mediated host immune response, and that co-transplantation of allogeneic non-parenchymal cells does not protect inoculated hepatocytes from rejection[6]. In an earlier study it was proposed that both liver parenchymal and non-parenchymal cells are required for spontaneous liver-induced tolerance[42]. On the other hand, a few published experiments revealed that a purified population of hepatocytes elicits a poor cytotoxic response[22, 23]. In addition, it has been suggested that purified hepatocytes, but not hepatic antigen-presenting cells, significantly mediate spontaneous liver-induced tolerance in the rat[48]. Thus, elimination of non-parenchymal cells may result in ablation of the immune response, which can be important in reducing *in vivo* alloreactivity.

Assessment of cell purity requires morphological and/or immunohistochemical techniques. In this study, the contamination of each preparation was determined by staining with monoclonal antibodies for the presence of antigens and markers specific to intrahepatic lymphocytes and non-parenchymal cells. By using these simple methods we demonstrated that Percoll purification significantly reduces the contamination of hepatocyte preparations by antigen-presenting cells. In this study, staining with the cell markers and flow cytometry analysis showed that there were less HLA-DR$^+$ (class II) cells, and only three other markers (CD34, CD68, and CD90) were detectable in all tested Percoll-purified cells. Further reduction in the expression of HLA-DR$^+$ cells was observed in cryopreserved/thawed hepatocyte preparations. The ability to cryopreserve hepatocytes which retain, after thawing, the functionality of freshly isolated cells would enable more widespread and effective use of these valuable cells. However, cryopreservation is associated with a loss in hepatocyte viability and plating efficiency. The mechanism of cell death during cryopreservation is incompletely understood, but may involve spontaneous apoptosis regulated by the Bcl-2 protein family[18, 47]. Apoptosis, identified as an important cause of death of freshly isolated and banked cells as well as of rejection of the transplanted allogeneic hepatocytes, deserves further investigative attention. In this study, the Bcl-2-related proteins tested in the isolated hepatocyte preparations were detected in one of five cases, which indicates that the applied hepatocyte cryopreservation techniques were sufficiently reproducible. Although our data illustrate the importance of human hepatocyte banking, the influence of prolonged storage of human hepatocytes in liquid nitrogen has not been tested. Only a few studies have dealt with human hepatocytes after cryopreservation, and to date there are no reports indicating that the length of storage effects post-thaw results or cell functional and morphological integrity. However, it is broadly accepted that the temperature at which frozen mammalian cells are stored affects the length of time after which the cells can be recovered. Thus, one of the major factors influencing successful hepatocyte cryopreservation is storage of the preparations at temperatures of –178°C to –190°C. Considering the very significant progress in human liver cell isolation, culture, and cryopreservation, it is clear that further investigation is needed to evaluate whether the length of cryostrorage can influence

hepatocyte survival and potential use of these valuable cells in the clinic.

In our experiments, the detection of $CD34^+$ small cells in hepatocyte preparations suggests that adult liver stem cells were harvested during cell isolation. According to recent reports, $CD34^+$ cells with hepatic stem-like properties can be isolated from the livers of patients with an extensive range of disease pathology. This includes patients transplanted for acute liver failure, severe alcoholic liver disease, and cirrhosis[43]. In our experiments, all processed livers originated from relatively healthy donors with no evident indications of liver insufficiency. The presence of $CD34^+$ cells isolated from such non-diseased livers indicates that these cells are widespread (albeit in relatively small numbers), and they offer a significant source of human liver stem cells for further studies. In this study, the hepatocyte preparations expressed very weak levels of chemokine receptors. Normal human liver contains a light infiltrate of lymphocytes, most of which express CXCR3, and some stain for CXCR4. Differences in lymphocyte populations can be attributed to differential expression of chemokines. Thus, CXCR4 is normally present on activated T cells and is dependant on interactions with SDF, whereas the expression of CXCR3-correlates with inflammatory responses[16, 25]. However, under non-inflammatory conditions only a faint expression of CXCR3 can be detected, which indicates again that in this study we were dealing with non-diseased livers.

Demonstrating that isolated cells retain various liver-specific functions would increase confidence in the clinical use of human hepatocytes. To determine whether the isolated hepatocytes maintained their differentiated phenotype, we tested several specific properties of cellular functional integrity. Hepatocyte energy status, evaluated by intracellular ATP concentration measurements, is a good indicator of hypothermic stress injury. The measurement of ATP content in a cell preparation is essential to ensure that hepatocytes are metabolically viable. Based on our results, the energy status evaluated by intracellular ATP concentration measurements was maintained in all the tested preparations. However, there was a considerable ATP loss in cryopreserved hepatocytes immediately after thawing, which is consistent with other reports[34]. The large standard deviations were expected and reflect the fact that the energy status varies dramatically among individuals. Based on our previous results we conclude that when cryopreserved hepatocytes are thawed and cultured briefly under optimized conditions, there is only a slight decrease in intracellular ATP content[36].

Other liver-specific functions of hepatocytes, as measured by their urea production, were maintained over the course of a 4-h incubation of cell suspensions. Urea synthesis is the pathway for the detoxification of ammonia, and such a positive response of isolated hepatocytes to ammonium chloride is a useful indicator of their functional integrity.

It is essential that hepatocytes isolated from non-transplantable livers express different cytochrome P450 (CYP450) functions. The long-term viability and functionality of isolated primary hepatocytes is evident from the expression of drug-metabolizing enzymes, the cytochromes P450. Hence, maintenance of hepatocellular metabolic functions and, in particular, gene expression of hepatic metabolizing enzymes is crucial. Although the metabolic activities of human hepatocytes were not tested in this study, we attempted to measure total CYP450 protein in different preparations of isolated cells. The spectrophotometric evidence reported in this paper seems to indicate that CYP450 protein is well preserved in all tested cells and is essentially identical in both freshly isolated and cryopreserved/thawed preparations. These measurements correspond well to the observations reported by other researchers[26, 29]. However, additional lots of human hepatocytes need to be tested in order to determine statistical significance.

Considering the large yield and vitality of cells isolated from livers not used for transplantation, primary human hepatocytes may be useful in clinical cell transplantation and in constructing a bioartificial liver device. Some recent clinical trials have shown that both freshly isolated and cryopreserved hepatocytes may be used to treat acute chronic liver insufficiency[2, 5, 7, 8, 21, 30]. The results of our study indicate that both freshly isolated hepatocytes and those recovered from cryopreservation display normal architecture at the light microscopic level and maintain various liver-specific functions *in vitro*. We found that some modifications in the hepatocyte preparations, such as depletion of non-parenchymal cells, further improves cell purity, which can be adapted to a further evaluation of hepatocyte immunogenicity. Although viable human hepatocytes can be isolated and cryopreserved on a routine basis, it is clear that strategies for the clinical application of hepatocyte transplantation remain to be outlined.

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