A QUANTITATIVE ANALYSIS OF LECTIN BINDING TO ADULT RAT HEPATOCYTE CELL SURFACES

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SUMMARY

A quantitative evaluation of lectin binding to adult rat hepatocyte cell surfaces was done using cells isolated by two different collagenase perfusion methodologies and cultured as monolayers with two different tissue culture media formulations (protocol I vs. protocol II). The presence of α-D-mannosyl and α-D-glucosyl groups was detected by the binding of Concanavalin A (Con A), Lens culinaris agglutinin (LCA), and Pisum sativum agglutinin (PSA) to freshly isolated cells. Furthermore, β-D-galactose (Ricinus communis agglutinin (RCA)) and sialic acid residues (wheat germ WGA) were also found. Protocols I and II served as models for evaluation of: a) the stripping effect of collagenase separation procedures, b) the restoration in culture of collagenase-stripped sugar residues, c) the effect of the culture environment on cell viability (as measured by lactic acid dehydrogenase (LDH) leakage) and the protein content of hepatocytes, and d) the presence of cell surface sugar residues as a function of culture duration. The ultrastructural morphology of freshly isolated and cultured hepatocytes was also evaluated. These studies indicated that a decline in lectin binding invariably occurred earlier than a massive leakage of LDH and a decrease in the protein content of the cells in culture. Ultrastructurally, autophagocytosis was an early phenomenon in cells isolated and cultured by protocol I, which was also inferior to protocol II regarding the preservation of hepatocyte glycocalyces. Sugar residues lost due to the collagenase-stripping effect were restored, as shown by lectin binding, within the first 24 h of culture. This stripping effect was confirmed by quantitative evaluations of lectin binding to hepatocytes in culture after an incubation with collagenase. This study shows that the binding of peroxidase-labeled lectins is a useful tool for quantitative evaluation of the sugar composition of hepatocyte cultures.

Key words: lectins; hepatocyte culture; glycocalyx; collagenase; autophagocytosis.

INTRODUCTION

The carbohydrate residues of hepatocyte plasma membrane glycoproteins, proteoglycans, and glycolipids are exposed only on the outer surface of the membrane where they serve as constituents of specific receptors for antibodies, hormones, and lectins (1–3). The hepatocyte glycocalyx is a dynamic structure whose components are in a constant state of turnover. Consequently, the cell surface oligosaccharides will suffer the primary impact of any proteolytic treatment used to separate the parenchymal cells from their original histologic associations (4). Furthermore, when hepatocytes are placed in an artificial milieu designed to maintain long-term functionality, their surfaces should reflect those particular environmental conditions that affect glycoprotein synthesis. In this context, although substantial evidence has been accumulated showing that a partial and transitory preservation of specific functions is possible in cultured hepatocytes (5–8), their in vivo phenotype invariably undergoes extensive modification as the cells adapt to culture (9,10). Several strategies to delay this in vitro simplification have been attempted with varying degrees of success (11–13). Few studies have addressed the quantification of sugar residues present on cell surfaces of hepatocytes in culture (14) and the related proposition that conditions that enhance preservation of sugar moieties on cell surfaces may also serve to maintain phenotypic expression.

The quantification of glycocalyx oligosaccharides can be achieved through the use of labeled lectins (15–17). In a previous light and electron microscopic study of adult rat hepatocytes in situ we detected a high content of α-D-glucosyl groups [strong binding of Concanavalin A (Con A), Lens culinaris agglutinin (LCA), and to a lesser degree Pisum sativum agglutinin (PSA)]. A β-D-galactose content in the bile canalicular domain was also demonstrated by the strong binding of Ricinus communis agglutinin (RCA) (18). In addition, a strong binding of native wheat germ agglutinin (WGA) (18) and a lack of binding of succinylated WGA (Mcmillan, unpublished observation) confirmed the high content of sialic acid reported by others (19).
We have assessed the presence of oligosaccharides in the glycocalyx of freshly isolated hepatocytes, as well as in cells growing in long-term primary cultures, using two methods for cell isolation and two different tissue culture media formulations (protocols I and II). A cytophotometric quantification of the binding of four lectins (Con A, LCA, RCA, WGA) was correlated with an evaluation of total cell protein content of the cultures, the lactic acid dehydrogenase leakage into the medium, and the ultrastructural morphology of the cells, with special emphasis on the presence of autophagosomal profiles.

Our results indicate that a loss of cell surface oligosaccharides occurred during both hepatocyte isolation procedures. This loss was greater with the Berry and Friend collagenase perfusion procedure (20) than with the Seglen method (21). A recovery of these sugar residues after 24 h in culture occurred with either modified Waymouth medium (MWM) or Chee's essential medium (CEM). There was an enhanced preservation of cell surface oligosaccharides when cells were grown in CEM as compared to MWM. CEM maintained the protein content of the cells and diminished the autophagocytosis that was evident in these primary cultures of hepatocytes. The loss of hepatocyte cell surface oligosaccharides seemed to precede the decrease in total protein content in the cells.

**MATERIALS AND METHODS**

**Hepatocyte isolation.** Two methods were used to isolate adult rat hepatocytes from 150 to 200 g male Wistar rats fed ad libitum with water and Lab Chow. Protocol I followed the collagenase technique of Berry and Friend (20) with a minor modification to include a 6-min preperfusion with calcium-free Hanks' balanced salt solution (HBSS). A nonrecirculating flow, via the inferior vena cava, with a solution consisting of 50 U/ml collagenase (Cooper Biomedical, Malvern, PA), 6 mM calcium chloride, and 0.2 mg/ml soybean trypsin inhibitor in HBSS was used in the perfusion step. This perfusion fluid also contained 10 mM calcium chloride, 10 mM HEPES buffer, and 50 mg/ml gentamicin sulfate. When the liver was sufficiently digested (9 to 12 min of perfusion), it was removed from the animal and the cells were gently dispersed into a spinner flask containing MWM 752/1 (GIBCO, Grand Island, NY), supplemented with 17 mM glucose and the same concentrations of gentamicin, insulin, dexamethasone, and HEPES buffer listed above. This cell suspension was incubated at 37°C for 25 min, filtered through a 255-mm nylon mesh (Tetko Inc., NY), and centrifuged at 20 x g for 5 min at 4°C. The cell pellets were washed twice with cold MWM and centrifuged as before. The final cell pellets were resuspended in MWM (4°C) supplemented as above, and maintained until further use (no more than 45 min).

Protocol II used Seglen's method for hepatocyte isolation (21) with preperfusion and perfusion steps done in situ rather than after removal of the liver. The yield of viable hepatocytes (trypan blue excluding cells) was approximately 0.41 ± 0.13 x 10⁶ cells/g of wet liver for protocol I (n = 20) and 0.77 ± 0.16 x 10⁶ cells for protocol II (n = 26). The percentage of viable cells was about 79% ± 4.3 for protocol I vs. 93.3% ± 3.2 for protocol II.

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**Biochemical Assays**

**Protein assay.** Total cell protein was measured by a modification of a Ponceau-S-protein micromethod (23). Hepatocytes in culture were detached by a rubber policeman. This procedure also detached the collagen substrate. Consequently, the protein values, for culture media and attached cells, were corrected for protein content of added FBS and collagen substrates. The percentage of protein attached was calculated by comparing readings of unknowns with values from a standard curve. The following formula was used for this calculation:

\[
\% \text{ protein attached} = \frac{\text{AP} - \text{CM}}{\text{TP}} \times 100
\]

where:

- \( \text{AP} \) = attached cell protein (mg) + protein of collagen substrate (mg)
- \( \text{CM} \) = protein of collagen substrate (mg)
- \( \text{TP} \) = total protein seeded (mg) (as inoculated cells)

**Lactate dehydrogenase assay.** Lactate dehydrogenase (LDH) was assayed separately in culture media and in hepatocytes (24). Samples were stored at room temperature overnight before assay, with no decrease in enzyme activity compared to fresh samples. Intracellular enzyme was released by lysing the cells in phosphate buffered saline containing 1% Triton X-100. Lactate dehydrogenase activity was proportional to cell number, and was assayed by the pyruvate to lactate reaction, monitoring the rate of absorbance decrease at 340 nm and 37°C (24). Activity was expressed in international units. Background LDH activity, due to FBS added to media (typically 50 IU/liter), was subtracted from the total LDH assayed in media. The percentage of LDH leakage was calculated as follows:

\[
\frac{(\text{LDH in culture media}) - (\text{LDH in media controls})}{100/(\text{LDH in culture media} + \text{attached cells})} \times (\text{LDH in media controls})
\]

**Morphological Studies**

**Fixation protocol.** Before lectin staining, freshly isolated and monolayer cultures of hepatocytes of various ages (ranging from 3 h to 14 d) were fixed for 1 h in 0.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) buffered with 0.15 M fluorescent