Perfusion Culture Improves the Maintenance of Cultured Liver Tissue Slices

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ABSTRACT

Cultured precision-cut liver tissue slices are useful for studying the metabolism and toxicity of xenobiotics in liver. They may also be used to investigate the behavior of and interaction between different cell types in an intact histo-architecture. Because cultured liver tissues undergo a loss of function and morphology because of their separation from the blood supply, we investigated changes in key protein marker expressions in parenchymal and non-parenchymal cells, as well as in the extracellular matrix (ECM) at different time points. We also compared conventional culture methods such as static and dynamic cultures with perfusion culture, which allows a continuous exchange of the culture medium. In conventional culture methods, the expression of vimentin and collagen type IV decreased after 5 h in the non-parenchymal cells and the ECM, respectively, whereas the hepatocyte nuclear factor 4 alpha (HNF4α) staining in the hepatocytes remained constant. In perfusion culture, on the other hand, vimentin, collagen type IV, and HNF4α staining were clearly detectable after 5 h. The histo-architecture obtained from perfusion culture was also more compact than those obtained from conventional culture methods. After 24 h, only the perfusion cultured sample retained protein marker expression in all components of the liver tissue. Our results suggest that, to develop improved culture techniques for liver slices, changes at the early time-points should be taken into consideration. Our results also show that culture techniques that enable a continuous exchange of the culture medium seem to be superior to static or dynamic cultures in terms of maintaining the protein expression and the histo-architecture.

INTRODUCTION

LIVER SLICE CULTURE is integral to liver tissue engineering. Organs are composed of various sets of cells arranged in specific architectural patterns. This spatial arrangement allows a reciprocal interaction between the different cell types so that the organ may perform its various functions. In liver tissue engineering, hepatocytes are the cells of interest because they are responsible for specific functions such as xenobiotic metabolism and toxicity, glucose storage, fatty acid metabolism, protein synthesis, and bile formation.

However, recent studies have shown that hepatocytes are difficult to culture, because they are unable to function and survive without supporting cells such as the endothelial cells and stellate cells.[1,2] Previous attempts to culture these cells as co-culture systems have shown that hormonal and cellular interactions are important for culturing functional hepatocytes. Cultured liver tissue could be a useful tool for investigating the interaction between and maintenance of cells in a normal histo-architecture.[3,4]

The long-term culture of precision-cut liver tissue slices has been employed for many applications.[5,6] It has mainly

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been used for testing the metabolism and toxicity of chemicals in the liver tissue.[7] In contrast to testing with isolated hepatocytes, liver tissue slices allow researchers to test the complete set of liver cells, such as hepatocytes, endothelial cells, Kupffer cells, lymphocytes, and stellate cells, within intact organ architecture. In addition to the cellular components, liver tissue also consists of extracellular matrix (ECM), which contributes to the maintenance and stability of the tissue. All these components have to be maintained in the long-term culture of liver tissue to prevent a reduction in function and morphological damage after the tissue's separation from the blood supply.

Various culture methods have been developed to achieve the survival of hepatocytes in tissue slices.[8] All of these methods aim to improve the mass transfer from the surface of the tissue into the inner volume. The conventional methods employed involve static culture or dynamic culture systems in which different types of culture vessels are rolled or shaken on movable platforms.[7,8] The culture medium is usually changed at intervals during the 24-h incubation period. The most important factor thought to impede long-term culture is the loss of a continuous supply of oxygen and nutrients to the tissue due to its separation from the blood vessels.[5] It can be assumed that, immediately after its separation from the blood supply, alterations in the various components such as parenchymal and non-parenchymal cells as well as in ECM are occurring in the cultured liver tissue, which is subsequently affecting the quality of the tissue slice. Therefore, we have investigated at different time points (5 h and 24 h of culture) whether first signs of alterations are occurring in the protein-expression profile of the parenchymal and non-parenchymal cells, as well as in the ECM. Such kind of assay, considering the different structural components found in the liver tissue, should reveal alterations in the cultured tissue in a sensitive fashion. In addition to the conventional culture methods such as static and dynamic culture, we used perfusion culture, allowing the continuous exchange of the culture media. Our results reveal that tremendous alterations in the protein marker expression are occurring after 5 h of culture using static and dynamic culture methods. Applying perfusion culture, we observed better maintenance of the cultured tissue.

MATERIALS AND METHODS

Preparation of liver slices

Male Han-Wistar rats weighing 250 to 300 g were obtained from the Center for Animal Resources (Singapore). The rats were anesthetized with sodium pentobarbital 40 to 50 mg/kg intraperitoneally. After laparotomy, the rat livers were removed, and cylindrical tissue cores of 8 mm in diameter were prepared. Liver slices (360 μm thickness) were obtained using a Krumdiek tissue slicer (Alabama Research and Development Corporation, Munford, AL).

Culture methods

The rat liver slices were subjected to 4 different culture conditions. In the first sample (Fig. 1A), the liver slices were cultured in multi-dish-well plates (Nunc, Roskilde, Denmark). In the second sample, the liver slices were cultured on polyester culture inserts (74-μm mesh size; Corning, NY) on a culture plate, which was placed on a rocker platform (Fig. 1B). The liver slices in the third sample were placed in a gradient culture container (Minucells and Minutissue, Bad Abbach, Germany), which has one inlet and one outlet each in the top and bottom compartments. The inlet and outlet in the bottom compartment were closed, and the sample was continuously exposed to flowing culture medium only on the top surface. The culture medium was transported using a peristaltic pump at a flow rate of 100 μL/min, without recirculation (Fig. 1C). In the fourth sample, the liver slices were cultured in a gradient culture container. In contrast to the third sample, these liver slices were exposed to flowing culture medium on the top and bottom surfaces (Fig. 1D). In conditions 1 and 2, Heptazyme-serum-free culture medium (Invitrogen Singapore Pte Ltd, Singapore) with 10^{-7} dexamethasone (Sigma-Aldrich, Singapore) and 1% antibiotic-antimycotic solution (Invitrogen Singapore Pte Ltd) was used. The experiments were run in a humidified atmosphere with 5% carbon dioxide at 37°C. In conditions 3 and 4, Heptazyme-serum-free culture medium (Invitrogen Singapore Pte Ltd) with 10^{-7} dexamethasone (Sigma-Aldrich), 1% antibiotic-antimycotic solution (Invitrogen Singapore Pte Ltd), and 6% HEPES buffer solution was used. The
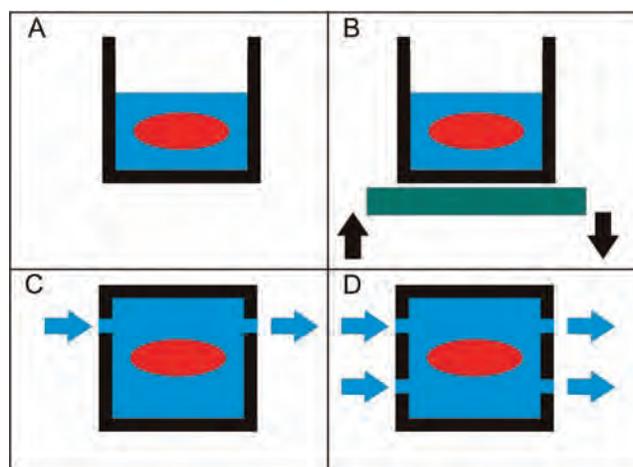
FIG. 1. Different culture techniques: Static culture (**A**), dynamic culture: rocker platform allows the movement of the culture media (**B**), single-sided perfusion culture: continuous exposure of the culture medium on the top surface of the tissue (**C**), double-sided perfusion culture: continuous exposure of the culture medium on both sides of the tissue (**D**). The culture media movement in culture techniques C and D enhanced mass transfer and the exchange of culture medium. Color images available online at www.liebertpub.com/ten.

experiments were run using a well-defined perfusion culture system at room atmosphere and at 37°C.[9,10]

Paraffin embedding and hematoxylin-eosin staining

After culturing, the liver slices were immediately fixed in 10% formalin, processed in increasing ethanol concentrations, and embedded in paraffin. Six-μm-thick cross-sections from the center of the tissue were selected and stained with hematoxylin-eosin.

Cryosectioning and immunohistochemistry

Eight-μm-thick cross-sections were prepared from the frozen liver slices using a cryostat CM 3050 S (Leica, Nussloch, Germany). Only representative tissue sections from the center of the slice were used. Immunolabeling was started by fixing the tissue in ice-cold ethanol for 10 min. After several rinses with phosphate-buffered saline (PBS), the sections were incubated with blocking solution containing PBS, 10% fetal calf serum, and 1% bovine serum albumin (BSA) for 30 min. The primary antibodies were incubated for 1.5 h in blocking solution. Hepatocyte nuclear factor 4 alpha (HNF4α) antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) was diluted at a ratio of 1:100, and the collagen type 4 antibody (Dr. Furthmayr, University of Iowa, Department of Biological Sciences, Iowa City, IA, under contract NO1-HD-7–3263 from the NICHD) was diluted at 1:50. The specimens were incubated for 45 min with donkey-anti-mouse–immunoglobulin G–fluorescein isothiocyanate–conjugated or donkey-anti-goat-IgG-FITC-conjugated secondary antibodies, which had been diluted at 1:200 in PBS containing 1% BSA (Jackson Immunoresearch Laboratories, West Grove, PA). Cy3-labeled vimentin was applied for 45 min and diluted at 1:400. The sections were then analyzed using an LSM 510 Meta microscope (Zeiss, Oberkochen, Germany). Images were taken with a digital camera and processed using Photoshop 5.5 (Adobe Systems, San Jose, CA).

Image processing

To quantitatively describe the dynamic process of the tissue culture under different conditions, each image is partitioned into "object" and "background" through threshold segmentation. For each image, a threshold T is applied to convert it to a binary image, in which a pixel is classified as object if its intensity is not less than t and as background otherwise. Because the background intensity in the original images may vary because of instrument baseline drift or immunostaining procedures, a threshold must be determined for each image. The Otsu method[11] is adopted to find the optimal threshold in this work. The procedure is briefly introduced here. For each possible threshold t, intra-group (object and background) variances are calculated. The t that minimizes the weighted sum of intra-group variances is the optimal threshold T. Because the threshold segmentation method used here processes each pixel independently,

grainy noise will affect segmentation. To remove the isolated pixel noise and join the separated portions of the features in the binary images, erosion and dilation[12] operation are applied to binary images from the segmentation.

Image quantification

In addition to signal intensity, areas of the objects (collagen and nuclei) were also used to characterize the images. Using area as a parameter, effects of variations in detector gain, offset, and immunostaining could be eliminated because the threshold was calculated for each image. The area of an object was obtained by counting the bright pixels in the binary image from the segmentation. In addition to total area and average intensity, the distribution of object size and signal intensity were calculated and plotted into histograms. To eliminate the effect of microscopic magnification, the total areas of the objects were normalized to the whole area of the image (i.e., collagen ratio and nuclei ratio were defined by the ratio of collagen area and nuclei area to the whole area of the image, respectively).

Protein measurement and sodium dodecyl sulfate-polyacrylamide gel electrophoresis and albumin immunoblotting

Six cultured liver slices of each culture set-up were homogenized in equal volume of a sample buffer containing 2% sodium dodecyl sulfate (SDS), 10% glycerin, 125 mM Tris-hydrochloric acid, 1 mM ethylenediaminetetraacetic acid (all obtained from Sigma-Aldrich-Chemie, Singapore) and centrifuged at $10,000 \times g$ for 10 min. The supernatants were used in the following experiments. The amount of proteins in each sample was determined using a protein micro-assay (Bio-Rad Laboratories, Hercules, CA). Protein samples were separated using SDS–polyacrylamide gel electrophoresis (PAGE) in 10% Laemmli mini-gels and Coomassie stained. In the case of the albumin immunoblotting, proteins were electrophoretically transferred to P-Immobilon membranes (Millipore, Eschborn, Germany). To detect albumin protein, the blots were blocked (PBS, pH 7.2; 0.05% Tween, Sigma, Deisenhofen, Germany; 10% horse serum, Boehringer, Mannheim, Germany) and then incubated for 1 h at room temperature with a rabbit polyclonal antiserum raised against albumin diluted 1:2000 (MP Biomedicals, Eschwege, Germany). An alkaline phosphatase-conjugated anti-rabbit immunoglobulin-antiserum (1:1000, Sigma, Singapore), applied for 45 min as described earlier, served as a detecting antibody. Blot development was started by addition of alkaline phosphatase substrate-chromogen (Zymed, San Francisco, CA). Washing the membrane in tap water stopped the reaction. Immunoblots were documented using a Scan Jet 6200 C (Hewlett Packard, Greeley, CO). Determination of apparent molecular weight was performed in conjunction with a broad-range molecular-weight protein standard (Bio-Rad Laboratories).
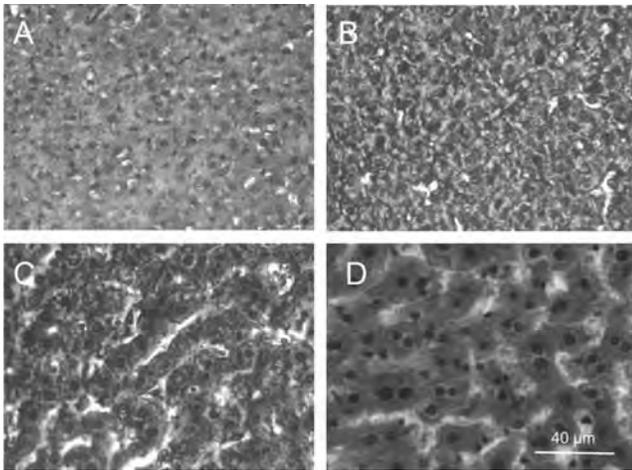
FIG. 2. Histomorphology of liver slice tissue cultured under static conditions at different time periods. Fresh isolated tissue (**A**) and tissue cultured for 5 h (**B**) revealed a compact histology. After 24 h, slightly increased spaces were found between the hepatocyte rows (**C**). The hepatocytes displayed normal morphologies and still appeared to be connected to each other. Similar results were also found after 7 days of culture (**D**).

RESULTS

Histomorphology in static culture

To compare our results with the results obtained by other investigators, we analyzed the histomorphological changes of the cultured tissue in hematoxylin-eosin-stained paraffin-embedded tissue. Freshly prepared liver slices showed a compact histo-architecture (Fig. 2A). Round-shaped cell nuclei were present in the hepatocytes, which line the sinusoids. Similar results were visible after 5 h of culture (Fig. 2B). After 24 h, the hepatocytes still displayed their hexagonal shape and appeared to be interconnected to each other (Fig. 2C). Some cell nuclei were more condensed than those in the freshly isolated tissue. The spaces between the hepatocyte rows were enlarged. Even after 7 days of culture, the cell shape of the hepatocytes and their arrangement in rows were preserved. The spaces between the hepatocyte rows were now found to be significantly enlarged, and more hepatocytes were multi-

nucleated (Fig. 2D). The histo-morphological analysis of paraffin-embedded tissue indicates only mild alterations in cultured tissue, even after 7 days of culture, consistent with similar observations previously reported.[5,13]

Protein marker expression in non-cultured liver tissue slices

To obtain information about the expression of cell-specific protein markers in the liver tissue slices, we investigated the protein marker expression *via* immunohistochemistry. Vimentin is found in the stellate cells and in the fibroblast representing the non-parenchymal cells.[14] HNF4α is a transcription factor expressed in the cell nuclei of hepatocytes, indicating their (maintained) differentiation status.[15] Collagen type IV is situated in the space of Disse, between the hepatocytes and the sinusoids.[16] In freshly prepared liver tissue slices, vimentin expression was predominately found in the stellate cells (Fig. 3A). Fine extensions of the cytoplasm were visible from the major body of the stellate cells. HNF4α staining was confined to the cell nuclei of the hepatocytes (Fig. 3B). The prominent collagen type IV staining indicated the location of the space of Disse and the staining surrounded the sinusoidal spaces (Fig. 3C).

Changes in the protein marker expression after 5 h in static-, dynamic-, and perfusion-cultured liver tissue slices

After 5 h of incubation under static conditions, there was a tremendous reduction in vimentin-positive cells (Fig. 4A). In contrast, dynamic-cultured tissue revealed many vimentin-stained cells with fine cell extensions (Fig. 4B). Neither single- nor double-sided perfusion cultured liver tissues displayed change in vimentin-staining (4C, D). It was also observed that the histo-architecture was more stable under perfusion-culture conditions (Fig. 4C, D). In contrast to the tremendous changes in vimentin-staining, HNF4α was expressed in all 4 culture conditions after 5 h (Fig. 4E-H). Representative staining profiles were further assessed using detailed image analysis (Fig. 5). The analysis consisted of the size distribution and signal intensity of the HNF4α staining in the hepatocyte nucleus. Fig. 5C shows distinct differences
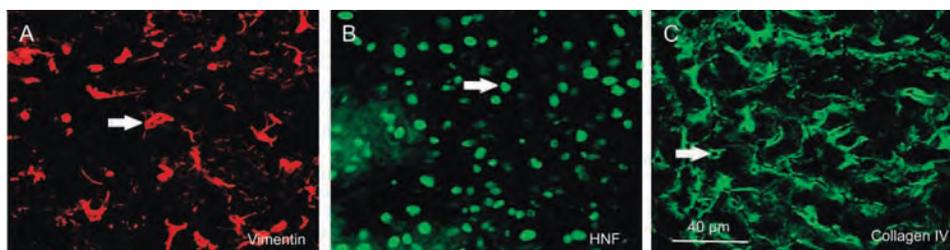
FIG. 3. Liver tissue before culture: Vimentin-staining of non-parenchymal cells, arrowed (**A**), hepatocyte nuclear factor (HNF4)alpha–staining of hepatocytes, arrow marks cell nucleus (**B**) and collagen type IV staining, arrow indicates sinusoidal space surrounded by collagen type IV positive fibers (**C**). Color images available online at www.liebertpub.com/ten.
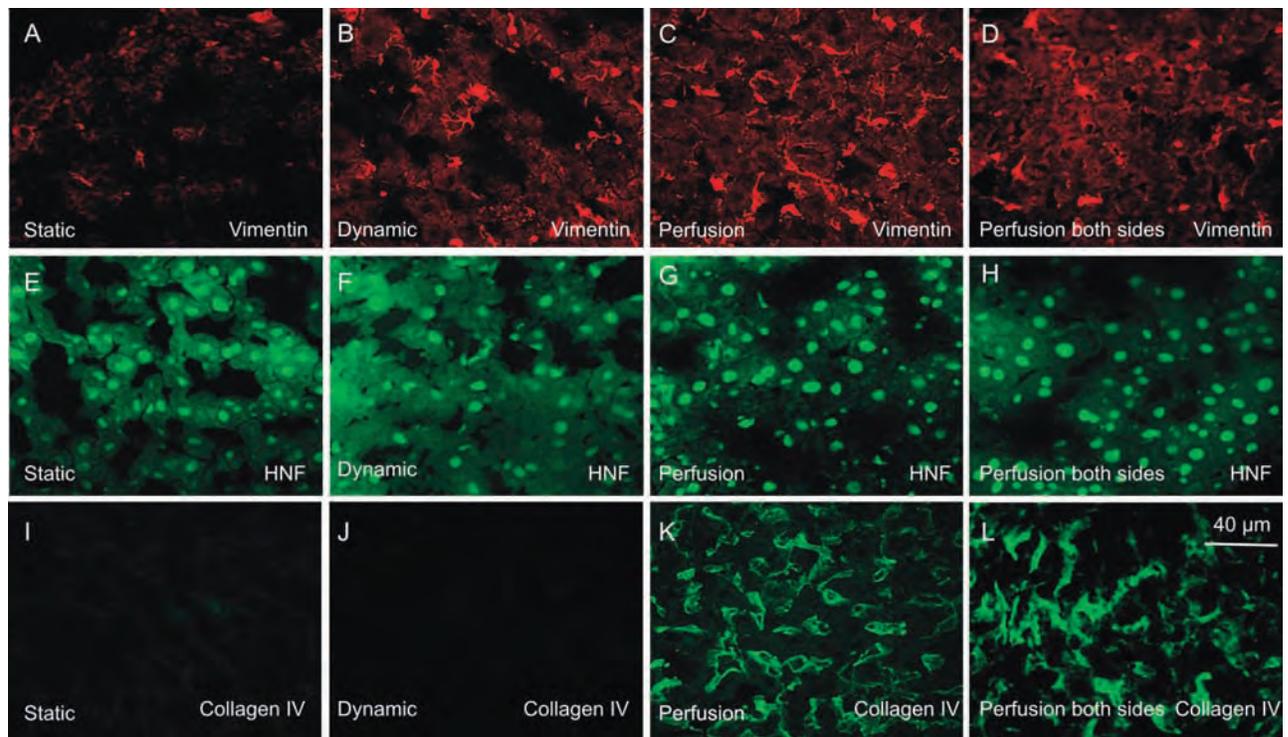
FIG. 4. Protein marker expression after 5 h of culture: Vimentin expression was reduced after static (**A**) and dynamic culture (**B**) but did not change after single-sided (top) perfusion culture (**C**) and double-sided perfusion culture (**D**). Extensive spaces between the hepatocyte rows appeared in static (**A**) and dynamic (**B**) cultured tissue (**B**). Hepatocyte nuclear factor (HNF) 4 alpha was observed in all applied culture methods (**E–H**). Collagen expression was absent in static (**I**) and dynamic culture (**J**) but was visible in single-sided (top) (**K**) and double-sided (**L**) perfusion culture. Color images available online at www.liebertpub.com/ten.

in the size distribution in the smaller areas (100 pixels) for the different culture conditions, whereas minor differences in HNF4α nuclear size distribution were found in other areas (200, 300, 400, and 500 pixels) (Fig. 5C). A comparison of the signal intensity of HNF4α staining in the hepatocyte nucleus revealed that staining profiles with high signal intensity were more frequently found in perfusion-cultured tissue than in static- or dynamic-cultured tissue (Fig. 5D).
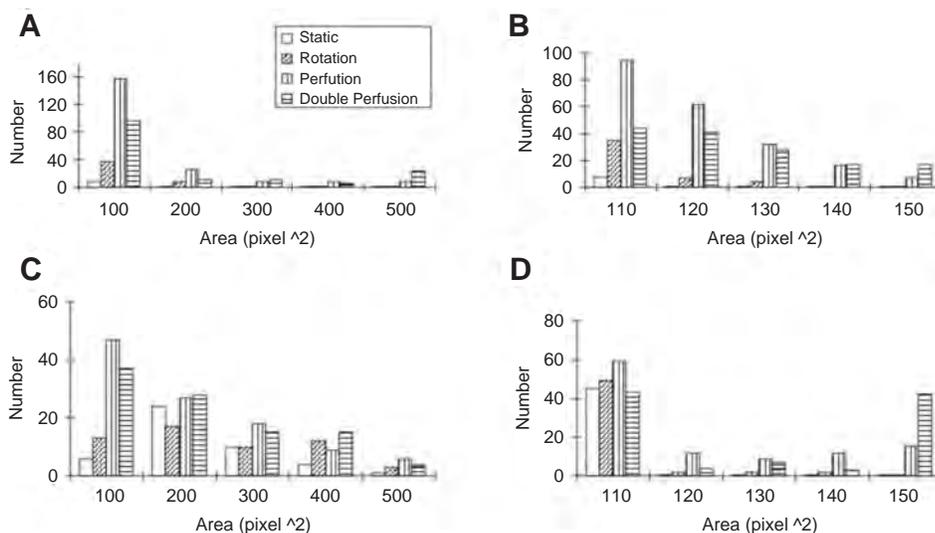
FIG. 5. Image analysis after 5 h of incubation: Different sizes (**A**) and intensities (**B**) of collagen expression were observed in the 4 applied culture conditions. Different sizes (**C**) and different intensities (**D**) of hepatocyte nuclear factor 4 alpha expression were analyzed in the 4 applied culture conditions.

Collagen type IV expression could not be detected after 5 h in static- and dynamic-cultured tissue (Fig. 4I, J). In contrast, collagen type IV expression was found surrounding the sinusoids in perfusion-cultured tissue (Fig. 4K, L). This indicates a well-maintained preservation of collagen type IV expression in the space of Disse and, therefore, an intact liver architecture in perfusion-cultured tissue after 5 h, unlike in the static- and dynamic-cultured tissue. The analysis of the different areas (Fig. 5A) and signal intensity (Fig. 5B) showed that, under perfusion-culture conditions, the expression of collagen type IV is distinctly maintained.

Changes in the protein-expression profile after 24 h in static-, dynamic-, and perfusion-cultured liver tissue slices

After 24 h of incubation under static (Fig. 6A) and dynamic (Fig. 6B) cultures, only few vimentin-positive stained cells were found. Single-sided (top) perfusion culture of the liver tissue obtained similar results as static and dynamic culture (Fig. 6C). However, double-sided perfusion culture of the liver tissue resulted in a distinct increase in stained cells (Fig. 6D). HNF4α staining was observed after 24 h of culture in all tested culture conditions (Fig. 6E-H). An image analysis of representative staining profiles revealed that,

under static conditions, HNF4α staining was found only in areas of 100 and 200 pixels (Fig. 7C). Perfusion culture led to staining in areas with 300, 400, and 500 pixels, whereas no staining was observed in these areas for static culture. Distinctly more cells with high HNF4α staining intensity were found in the double-sided perfusion-cultured tissues than in the other tissues (Fig. 7D). Collagen type IV expression was low in static, dynamic, and single-sided perfusion culture tissues (Fig. 6 I-K). A faint staining pattern was observed in the double-sided perfusion culture tissues (Fig. 6L), and image analysis showed that collagen type IV staining was only detected in small areas (100 pixels) (Fig. 7A).

Protein concentration and albumin content of differently cultured liver tissue slices

Because a non-circulatory approach was selected in the perfusion culture set-up, resulting in different amounts of used culture media, a comparative approach to investigate the amount of protein secreted in the culture medium supernatant was difficult to apply. Therefore, we analyzed the average protein concentration and the albumin content in the cultured liver tissue slices (Fig. 8). It was observed that the average protein content in the rocker and perfusion set-up was up to 30% higher then in the static culture set-up
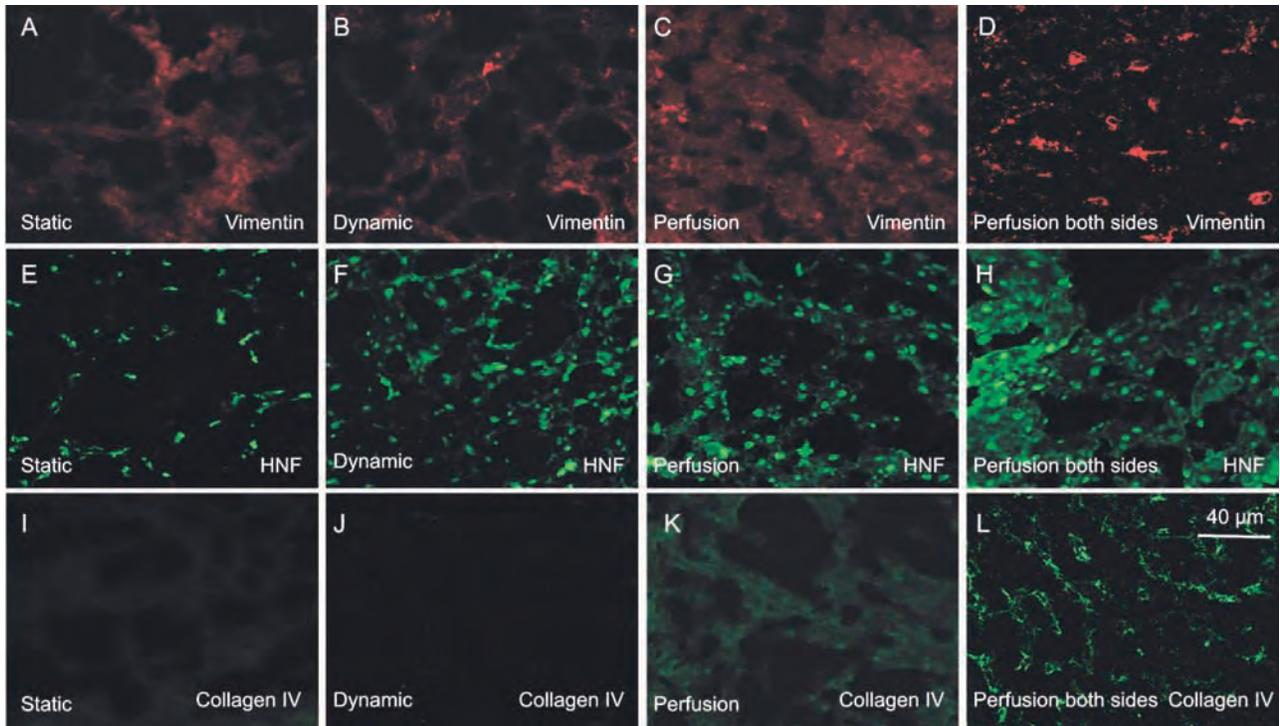
FIG. 6. Protein marker expression after 24 h: Static culture (**A**), dynamic culture (**B**), and single-sided perfusion culture (**C**) led to no visible vimentin staining. Only double-sided perfusion culture (**D**) resulted in a visible vimentin staining. Hepatocyte nuclear factor 4 alpha (HNF4α) expression could be observed in all applied culture conditions (**E–H**). Static culture (**I**), dynamic culture (**J**), and single-sided perfusion culture (**K**) resulted in an absence of collagen expression. Only double-sided perfusion culture (**L**) revealed a faint collagen expression. Color images available online at www.liebertpub.com/ten.
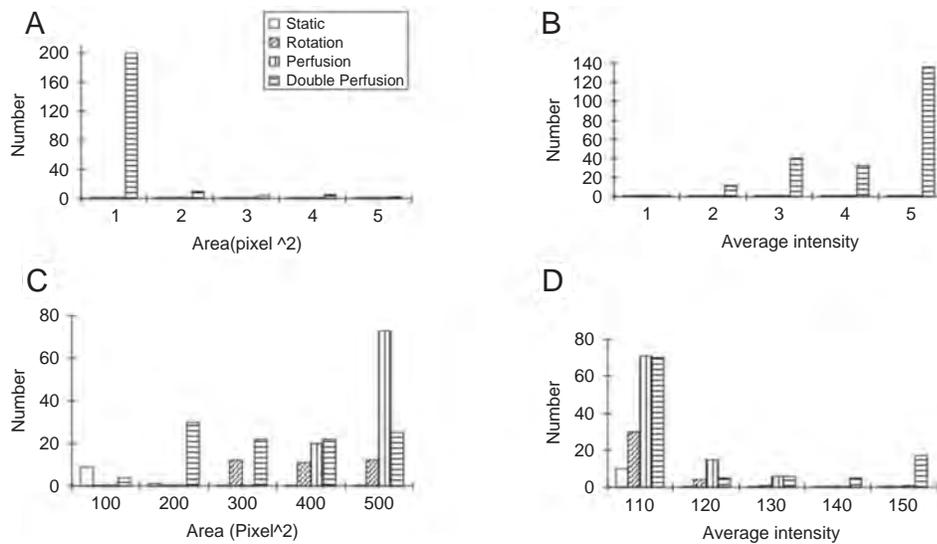
FIG. 7. Image analysis after 24 h of incubation: Different sizes (**A**) and different intensities (**B**) of collagen expression were shown in the 4 applied culture conditions. Different sizes (**C**) and different intensities (**D**) of hepatocyte nuclear factor 4 alpha expression were analyzed in the 4 applied culture conditions.
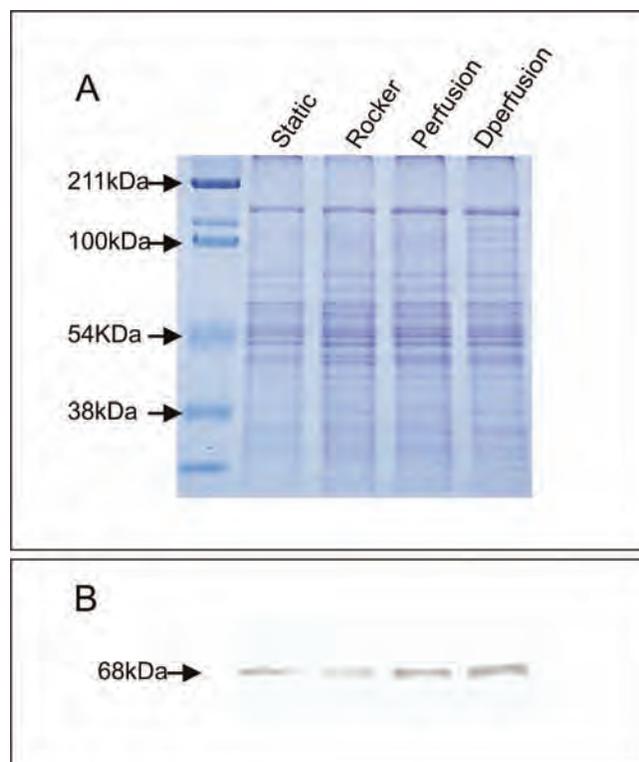
FIG. 8. Protein concentration and western blotting results of differently cultured liver slices. (**A**) Coomassie staining of a gel showing liver slices after 24 h of culture lysed in equal sample buffer solutions. No differences in protein band pattern could be observed in the differently cultured liver slices. Higher average protein concentration was observed in rocker and both perfusion conditions than in the static condition. (**B**) Representative immunoblotting result revealed that albumin content is higher in perfusion culture technique than in static and rocker culture. Color images available online at www.liebertpub.com/ten.

(Fig. 8A), whereas no changes in the band pattern was apparent. In addition, higher albumin content was found in both perfusion conditions than with the static and rocker culture technique (Fig. 8B).

DISCUSSION

The long-term culture of liver tissue slices is a commonly used technique to investigate the toxic effects of drugs or to study drug metabolism in hepatocytes.[7] After separation from the blood supply, the tissue slices can only be supplied with nutrients through their surface, and hence a continuous process of tissue degradation sets in. Because the hepatocytes perform most of the important functions of the liver, many studies focus on the survival and functional parameters of hepatocytes in cultured liver tissue. Lactate dehydrogenase leakage, histomorphology of paraffin-embedded tissue, and expression of functional enzymes (e.g., different types of cytochromes) are generally used to assess the maintenance of the hepatocytes.[13,17] In addition, most of the published data address longer periods of culture, ranging from 3 to 10 days.[5,13] The aim of this investigation was to establish an assay that is reflective of the functional and morphological status of all liver tissue components in culture. The studies were done at 5 and 24 h of culture. We used immunohistochemistry to monitor the expression of relevant proteins in parenchymal and non-parenchymal cells, as well as in the ECM. The reduced protein marker expression should indicate the loss of function and changes in the phenotype. The immunohistochemistry was conducted in cryosections to ensure the accessibility of the investigated antigens and to investigate non-fixed tissue.

To compare our results with the data in the available literature,[13,18] we cultured liver tissue slices under static conditions for 0, 5, and 24 h and 7 days. After culturing, the tissue was immediately embedded in paraffin and stained with hematoxylin and eosin (Fig. 2). Microscopical analysis revealed minor changes in the morphology of the cultured tissue. In particular, the hepatocytes maintained their cell shape well, and the spaces between the rows of hepatocytes were only slightly enlarged after 24 h and 7 days of culture. In contrast, the analysis of the cryosections revealed a significantly larger space between the hepatocytes rows than in freshly isolated tissue after only 5 h of culture (Fig. 4a and 4E). This suggests that the cultured tissue's micro-architecture had already started to deteriorate. In addition, the results indicate that the investigation of cryosectioned tissue could reveal early signs of structural changes in cultured tissue, whereas the histo-morphological assessment of paraffin-embedded tissue could not.

HNF4α is a central regulator of hepatocyte functions and differentiation.[15] Therefore, a reduction in HNF4α protein expression would mean deteriorating control of the hepatocyte functions. HNF4α staining is found to be expressed after 5 h in all applied culture conditions. After 24 h of culture, however, the expression profile is different for all 4 culture conditions. The image analysis showed that the most severe changes occurred in the static culture and that the HNF4α expression is better preserved in perfusion culture. A significant reduction of vimentin staining in the static-cultured liver tissue and a loss of collagen expression in the static- and dynamic-cultured liver tissue was observed after 5 h of culture. The vimentin and collagen expression remained steady in terms of staining intensity and localization for the perfusion-cultured tissues. After 24 h of culture, only the double-sided perfusion-cultured tissue displayed vimentin staining and collagen expression. These results indicate that degradation processes occur much faster in the non-parenchymal cells and in the ECM than in the hepatocytes. It can be assumed that hepatocytes in cultured tissue slices can maintain their functions and phenotype after 5 h, despite the tremendous morphological changes taking place in the non-parenchymal cells and in the surrounding ECM. However, changes in the hepatocyte differentiation were observed after 24 h. The loss of support from the non-parenchymal cells and the ECM may cause this. Because the collagen type IV molecules in the space of Disse contribute to the mechanical stability of the liver parenchyma, a reduction in collagen type IV expression would also explain the rapid formation of significant spaces in static and dynamic culture conditions. In future experiments, we would like to investigate which specific signaling molecules are altered, causing a reduction in the functions and morphological changes in cultured tissue.

Under static culture conditions, culture medium that is neither moved nor refreshed surrounds the liver tissue slice. Therefore, static culture of liver tissue slices has been applied mostly in short-term studies (<3 h). For long-term culture, more sophisticated incubation systems in the form of dynamic culture conditions are needed. We selected the rocker platform as a representative culture method. This approach does not allow the exchange of culture medium but causes the movement of the culture medium. Applying our assay to this culture method revealed that, in dynamic culture, the protein marker expression can be maintained longer than in static culture. However, alterations in the non-parenchymal cells and in the ECM are clearly detectable in dynamic-cultured tissue after 5 h. Perfusion of precision-cut liver slices was performed in short-term studies of hormone-regulated hepatic glucose metabolism, and this approach has been shown to be useful for this application[19] but not investigated in culture of up to 24h. We applied a non-recirculatory perfusion system that allowed not only the movement, but also the exchange of the culture media. After 5 h of culturing, in the liver slices in the single-sided perfusion-culture system, which exposed the top surface of the tissue to a continuous flow of culture medium, a significant improvement in the maintenance of the cultured tissue was observed. It has been demonstrated that shear stress can affect the functions of isolated hepatocytes,[20–23] and it can be assumed that shear stress will also play a role in liver tissue culture. Perfusion culture systems will be in general associated with this drawback, and it requires the balance between shear stress and mass transfer that our own observations here and observations from other investigators[24] that the outer zone of the cultured liver slice is better preserved morphologically than the inner zone reflect. This method could not sustain the protein marker expression in all components of the cultured liver tissue after 24 h. A slight to mediocre improvement was obtained after 24 h when the tissue was cultured in the double-sided perfusion system (i.e., both sides of the tissue were perfused with fresh culture medium). Investigating the average protein concentration revealed no differences in the protein band pattern (Fig. 8A), but protein measurement showed a higher average protein concentration in the rocker and both perfusion conditions than in the static condition. The albumin content (indicator of synthetic functions) of the liver tissue slice after perfusion culture was higher than in static and rocker culture (Fig. 8B).

These results suggest that, to improve the culture conditions of liver tissue, the culture medium not only has to be moved, but also needs to be refreshed. This method also simulates the *in vivo* conditions better, which probably explains this significant improvement. It remains to be investigated whether the improved morphology and the improved maintenance of the protein expression can be incorporated into a small-scale circulatory system to study the metabolism and the detoxification processes using liver slice culture.

ACKNOWLEDGMENTS

REFERENCES

1. Bhatia, S. N., Yarmush, M. L. and Toner, M. Controlling cell interactions by micropatterning in co-cultures: hepatocytes and 3T3 fibroblasts. J Biomed Mater Res **34,** 189, 1997.

2. Chia, S. M., Lin, P. C. and Yu, H. TGF-beta1 regulation in hepatocyte-NIH3T3 co-culture is important for the enhanced hepatocyte function in 3D microenvironment. Biotechnol Bioeng **89,** 565, 2005.

3. Bedossa, P. and Paradis, V. Liver extracellular matrix in health and disease. J Pathol **200,** 504, 2003.

4. Moronvalle-Halley, V., Sacre-Salem, B., Sallez, V., Labbe, G. and Gautier, J. C. Evaluation of cultured, precision-cut rat liver slices as a model to study drug-induced liver apoptosis. Toxicology **207,** 203, 2005.

5. Lerche-Langrand, C. and Toutain, H. J. Precision-cut liver slices: characteristics and use for *in vitro* pharmaco-toxicology. Toxicology **153,** 221, 2000.

6. Vickers, A. E. and Fisher, R. L. Organ slices for the evaluation of human drug toxicity. Chem Biol Interact **150,** 87, 2004.

7. Martin, H., Bournique, B., Blanchi, B. and Lerche-Langrand, C. High-throughput optimization by statistical designs: example with rat liver slices cryopreservation. Anal Biochem **319,** 56, 2003.

8. Olinga, P. *et al.* Comparison of five incubation systems for rat liver slices using functional and viability parameters. J Pharmacol Toxicol Methods 38, 59, 1997.

9. Minuth, W. W. *et al.* Modulation of cell differentiation in perfusion culture. Exp Nephrol **7,** 394, 1999.

10. Schumacher, K., Strehl, R. and Minuth, W. W. Urea restrains aldosterone-induced development of peanut agglutinin-binding on embryonic renal collecting duct epithelia. J Am Soc Nephrol **14,** 2758, 2003.

11. Otsu, N. A threshold selection method from gray level histograms. IEEE Trans Syst Man Cybern **9,** 62, 1979.

12. Serra, J. Image Analysis and Mathematical Morphology. New York: Academic Press, 1982.

13. Nonaka, H. *et al.* Development of highly functional long-term culture method of liver slice embedded in agarose gel for bioartificial liver. Cell Transplant **12,** 491, 2003.

14. Zeilinger, K. *et al.* Time course of primary liver cell reorganization in three-dimensional high-density bioreactors for extracorporeal liver support: an immunohistochemical and ultrastructural study. Tissue Eng **10,** 1113, 2004.

15. Odom, D. T. *et al.* Control of pancreas and liver gene expression by HNF transcription factors. Science **303,** 1378, 2004.

16. Amenta, P. S. and Harrison, D. Expression and potential role of the extracellular matrix in hepatic ontogenesis: a review. Microsc Res Tech **39,** 372, 1997.

17. Martin, H. *et al.* Induction of cytochrome P450 2B6 and 3A4 expression by phenobarbital and cyclophosphamide in cultured human liver slices. Pharm Res **20,** 557, 2003.

18. Hashemi, E., Dobrota, M., Till, C. and Ioannides, C. Structural and functional integrity of precision-cut liver slices in xenobiotic metabolism: a comparison of the dynamic organ and multiwell plate culture procedures. Xenobiotica **29,** 11, 1999.

19. McKee, R. L. *et al.* Perifused precision-cut liver slice system for the study of hormone-regulated hepatic glucose metabolism. J Pharmacol Methods **19,** 339, 1988.

20. Kan, P., Miyoshi, H. and Ohshima, N. Perfusion of medium with supplemented growth factors changes metabolic activities and cell morphology of hepatocyte-nonparenchymal cell coculture. Tissue Eng **10,** 1297, 2004.

21. Kan, P., Miyoshi, H., Yanagi, K. and Ohshima, N. Effects of shear stress on metabolic function of the co-culture system of hepatocyte/nonparenchymal cells for a bioartificial liver. Asaio J **44,** M441, 1998.

22. Koebe, H. G., Wick, M., Cramer, U., Lange, V. and Schildberg, F. W. Collagen gel immobilisation provides a suitable cell matrix for long term human hepatocyte cultures in hybrid reactors. Int J Artif Organs **17,** 95, 1994.

23. Tilles, A. W., Baskaran, H., Roy, P., Yarmush, M. L. and Toner, M. Effects of oxygenation and flow on the viability and function of rat hepatocytes cocultured in a microchannel flat-plate bioreactor. Biotechnol Bioeng **73,** 379, 2001.

24. Shigematsu, A., Motoji, N., Momose, Y., Iida, A. and Higashi, N. Viability of liver slices exhibiting absorption, metabolism, and elimination of substrates in culture medium. Exp Mol Pathol **69,** 119, 2000.

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