Biochemical and Biophysical Research Communications **274,** 1–3 (2000)
doi:10.1006/bbrc.2000.2912, available online at http://www.idealibrary.com on IDEAL®

BREAKTHROUGHS AND VIEWS

Recent Advances in Human Hepatocyte Culture Systems

Dieter Runge,*,†,[1] George K. Michalopoulos,†,[2] Steven C. Strom,† and Dorothee M. Runge*,†,[1]
**Klinik für Innere Medizin I, Martin Luther Universität Halle-Wittenberg, 06097 Halle, Federal Republic of Germany; and †Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261*

Received May 19, 2000

BACKGROUND

The liver fulfills many vital processes in mammals. It is the central organ of energy metabolism, biotransformation of xenobiotics, and synthesis of plasma proteins under physiological and pathophysiological conditions. Primary cultures of hepatocytes have been and still are an important tool to study liver-specific processes and functions. So far, most of the studies with hepatocyte cultures have primarily been performed with rat hepatocytes. However, interspecies differences in all aspects of hepatocyte function exist and are recognized and investigated since more than 30 years: Differences have been reported for the intracellular distribution of gluconeogenic enzymes (1–4), for apolipoprotein A-IV expression (5), metabolic regulation of cholesterol and triacylglycerol synthesis (6), and cytochrome P450 induction (7, 8). Therefore, primary human hepatocytes should be the system of choice for the evaluation of liver specific processes in human: (i) the biology of human viral pathogens or parasites and (ii) drug metabolism in phase I and II reactions (9, 10).

MODEL SYSTEMS

Since human hepatocytes are available only in limited number, the development of culture systems that allow cultivation of differentiated and functional hepatocytes is of great importance. Several different attempts have been taken using different substrates (11), media and hormonal conditions usually in the initial or ongoing presence of serum (12, 13). Recently, a serum-free and chemically defined culture system has been established (14). In contrast to the more conventional two dimensional cultures three dimensional collagen gel sandwich cultures (12, 15, 16) or mixed cultures with the presence of non-parenchymal cells (17) were evaluated for prolonged hepatocyte function. A different report describes a system that involves the transfer of cells from a conventional two-dimensional to a three-dimensional collagen gel sandwich system (18). Culture systems that promote a long-term cultivation of human hepatocytes usually rely on the presence of serum (12) and/or the addition of growth factors like HGF, EGF, and Insulin (12, 14, 15, 19). The benefits of HGF addition to the culture medium is somewhat controversial. In short-term serum-treated cultures (up to 4 days) low concentrations of HGF mediated a down regulation of cytochrome P4501A1/2, and CYP3A4 protein and activity, while UDP-glucoroyltransferase and glutathione S-transferase activity were not modified (13). In contrast to these data, a more recent study demonstrated the maintenance of basal and inducible cytochrome P450 protein expression and enzymatic activity for 4–6 weeks even in the presence of high HGF concentrations (14).

A majority of studies with a pharmacological–toxicological objective depend on differentiated hepatocytes. Most investigators analyze the induction and activity of cytochrome P450 enzymes, especially the CYP 1A and 3A isoforms (7, 8, 12–14, 16, 19–23), fewer reports focus on phase II detoxifying enzymes (11, 13, 16). These studies used mostly short-term cultures, in which the cells were maintained for maximally 8 days. It is well known that hepatocytes require an adjustment period of about 4 days before full responsiveness to applied hormonal stimuli is observed in terms of cytochrome P450 induction (24). Therefore, the use of long-term human hepatocyte cultures is an important tool for pharmacological and toxicological studies. The recent development of two dimensional long-term culture systems in which hepatocyte morphology and function is maintained for several weeks (14, 19) allows

[1] Present address: Ingenium Pharmaceuticals AG, 82152 Martinsried, Federal Republic of Germany.
[2] To whom correspondence should be addressed at Department of Pathology, University of Pittsburgh, South 410 BST, Pittsburgh, PA 15261. Fax: 001-412-648-9846. E-mail: michalopoulos@msx.upmc.edu.

0006-291X/00 $35.00

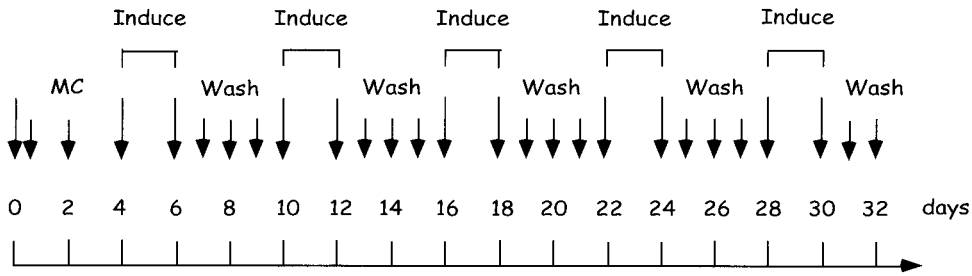

FIG. 1. Long-term cultures as a tool for screening in drug metabolism using noninvasive assays. Human hepatocytes can be cultured for several weeks. Prototypical inducers are applied for 48 h to increase enzymes involved in drug metabolism. Noninvasive assays are performed at the end of induction period, followed by wash-out periods of 3–4 days to downregulate enzyme levels again. Two hundred molecules (or more) may be tested in duplicate in the same culture (1 billion cells). MC, medium change. Adapted from Ref. 35.

the establishment of screening systems for cytochrome P450 inducers and to investigate drug-drug interactions. These culture systems should allow repeated treatment/wash-out cycles to screen for drug activity especially if well-established noninvasive assays as for testosterone-6β-hydroxylase (CYP3A4), ethoxyresorufin-deethylase (CYP1A) or chlorzoxazone-6-OH-hydroxylase (CYP2E1, D.M.R., unpublished observations) can be used to determine enzymatic activities (Fig. 1).

PROPAGATION OF HUMAN HEPATOCYTES FOR CELL TRANSPLANTATION AND GENE THERAPY

A different application of human hepatocyte cultures is represented by an increasing number of studies that used human hepatocyte either for therapeutical (25) or experimental cell transplantation studies (26). Here hepatocytes were cultured in the presence of serum, which allows proliferation to accomplish a stable gene transfer by retroviral vectors. In the first case a patient with familial hypercholesterolemia was treated by transplantation of autologous hepatocytes after her own hepatocytes had been modified *ex vivo* by retroviral transfer of the LDL-receptor gene (25). To overcome the critical shortage of human hepatocytes Kobayshi and co-workers (26) generated a reversible immortalized human hepatocyte cell line, which expresses some but not all of the investigated liver-specific markers. This method allowed amplification of the dedifferentiated cells with subsequent and complete excision of the immortalizing gene by Cre/Lox site specific recombination, which then led to reexpression of the investigated differentiation markers.

CULTURE OF HUMAN HEPATOCYTES IN BIOARTIFICIAL LIVER SUPPORT SYSTEMS

The use of human cells in bioartificial liver support systems is now under investigation (27, 28). In earlier applications these devices have utilized pig hepatocytes (29). This holds the potential danger of transmitting human pathogenic substances like PERV (porcine endogenous retrovirus) to the patient during treatment (30). Therefore, the efficiency of these systems is currently tested with human hepatocytes in co-culture with non-parenchymal cells, originally described by Guillouzo and coauthors (17). Promising results have been demonstrated investigating albumin secretion and basal cytochrome P450 activity for up to two weeks, using human hepatocytes alone in the liver support device. When hepatocytes were cocultured with non-parenchymal cells (approximately 17% of total number of cells) albumin synthesis increased about 10-fold, basal lidocaine metabolism 3-fold compared to hepatocyte monocultures. Furthermore, a significant reduction of LDH release was observed in cocultures compared to hepatocytes alone (28).

FUNCTIONAL ASSAYS TO CHARACTERIZE HEPATOCYTE-SPECIFIC FUNCTIONS

Which parameters should be used to evaluate the functionality of a given human hepatocyte culture system? Usually, maintenance of liver-specific functions is monitored by the expression of serum proteins and/or drug metabolizing enzymes (7, 8, 12, 14, 16–21, 26, 28, 29, 31, 32). Expression of these proteins is regulated by liver-enriched transcription factors C/EBPα, HNF-3, and HNF-4. In serum-free cultures expression and activity of these transcription factors was maintained for several weeks and is held responsible for maintenance of hepatocyte specific gene expression (31, 32). Serum protein expression is either monitored by RT–PCR or Northern blot analysis of mRNAs for albumin, transferrin, apolipoproteins or α-1-antitrypsin or by immunological detection of the corresponding proteins in the culture medium (12, 17–19, 28, 29, 31, 32). In addition, expression or inducibility of phase I or phase II drug metabolizing enzymes is often used as a convenient marker for hepatocyte specific functions (7, 8, 12–14, 16, 19–24, 28, 29). These basal cellular functions, i.e., synthesis of mRNA that are normally abundant in functional primary cells or the corresponding synthesis

of abundant proteins seems to be maintained for weeks or even months. However, the quality of these markers as parameters for hepatocyte specific function is arguable for two reasons: First, the detection of a certain mRNA by itself, especially by RT–PCR, does not necessary indicate the presence of the corresponding protein. Second, even if the protein is detectable by Western blot analysis, one cannot draw any conclusion on its functionality, because cofactors required for enzymatic activities might be missing. This has been demonstrated by our group: although the total amount of inducible cytochrome P450 protein varied only slightly between earlier and later time points in the human hepatocyte cultures, the corresponding inducible enzymatic activity did not correlate with steady-state protein levels in all cases. It declined during the culture, probably due to the loss of the heme component (14). Therefore, hepatocyte function should be demonstrated by more sensitive metabolic or enzymatic activities, or, if membrane transport proteins are investigated, transport assays of model substances (e.g., rhodamine 123) should be performed.

Recently, improvements have been made in hepatocyte cryoconservation techniques (33, 34). This may allow to establish cell banks, that should improve the availability of human hepatocytes for cell transplantation, bio-artificial liver support, as well as pharmacological studies.

REFERENCES

1. Ballard, F. J., and Hanson, R. W. (1969) *J. Biol. Chem.* **244,** 5625–5630.
2. Brech, W., Shrago, E., and Wilken, D. (1970) *Biochim. Biophys. Acta* **201,** 145–154.
3. Elliott, K. R., and Pogson, C. I. (1977) *Biochem. J.* **164,** 357–361.
4. Hod, Y., Utter, M. F., and Hanson, R. W. (1982) *J. Biol. Chem.* **257,** 13787–13794.
5. Elshourbagy, N. A., Walker, D. W., Bougusky, M. S., Gordon, J. I., and Taylor, J. M. (1986) *J. Biol. Chem.* **261,** 1998–2002.
6. Lin, B. Y., Vonk, R. L., Slooff, M. J. H., Kuipers, F., and Smit, M. J. (1995) *Brit. J. Nutr.* **74,** 197–207.
7. Kocarek, T. A., Schuetz, E. G., Strom, S. C., Fisher, R. A., and Guzelian, P. S. (1995) *Drug. Metab. Dispos.* **23,** 415–421.
8. Silva, J. M., Morin, P. E., Day, S. H., Kennedy, B. P., Payette, P., Rushmore, T., Yergey, Y. A., and Nicoll-Griffith, D. A. (1998) *Drug Metab. Dispos.* **26,** 490–496.
9. Moshage, H., and Yap, S. H. (1992) **15,** 404–413.
10. Li, A. P., Maurel, P., Gómez-Lechón, M. J., Cheng, L. C., and Jurima-Romet, M. (1997) *Chem.–Biol. Interact.* **107,** 5–16.
11. Kane, R. E., Li, A. E., and Kaminski, D. R. (1995) *Drug. Metab. Dispos.* **23,** 303–307.
12. Kono, Y., Yang, S., and Roberts, E. A. (1997) *In Vitro Cell. Dev. Biol.* **33,** 467–472.
13. Donato, M. T., Gómez-Lechón, M. J., Jover, R., Nakamura, T., and Castell, J. V. (1998) *J. Pharmacol. Exp. Ther.* **284,** 760–767.
14. Runge, D., Köhler, C., Kostrubsky, V. E., Jäger, D., Lehmann, T., Runge, D. M., May, U., Beer Stolz, D., Strom, S. C., Fleig, W. E., and Michalopoulos, G. K. Submitted.
15. Michalopoulos, G. K., Bowen, B., Nüssler, A. K., Becich, M. J., and Howard, T. A. (1993) *J. Cell. Physiol.* **156,** 443–452.
16. Kern, A., Bader, A., Pichelmayr, R., and Sewing, K. F. (1996) *Biochem. Pharmacol.* **54,** 761–772.
17. Clement, B., Guguen-Guillouzo, C., Campion, J. P., Glaise, D., Bourel, M., and Guillouzo, A. (1984) *Hepatology* **4,** 373–380.
18. Chen, H. L., Wu, H. L., Fon, C. C., Chen, P. J., Lai, M. Y., and Chen, D. S. (1998) *J. Biomed. Sci.* **5,** 435–440.
19. Ferrini, J. B., Pichard, L., Domergue, J., and Maurel, P. (1997) *Chem.–Biol. Interact.* **107,** 31–45.
20. Langouët, S., Coles, B., Morel, F., Beaune P., Guengerich, F. P., Ketterer, B., and Guillouzo, A. (1995) *Cancer Res.* **55,** 5574–5579.
21. Chang, T. K. H., Yu, L., Maurel, P., and Waxman, D. J. (1997) *Cancer Res.* **57,** 1946–1954.
22. Li, A. P., Reith, M. K., Rasmussen, A., Gorski, J. C., Hall, S. D., Xu, L., Kaminski, D. L., and Cheng, L. K. (1997) *Chem.–Biol. Interact.* **107,** 17–30.
23. Guillén, M. I., Donato, M. T., Jover, R., Castell, J. V., Fabra, R., Trullenque, R., and Gómez-Lechón, M. J. (1998) *J. Pharmacol. Exp. Ther.* **285,** 127–134.
24. Liddle, C., Goodwin, B. J., George, J., Tapner, M., and Farrrel, G. C. (1998) *J. Clin. Endocrinol. Metab.* **83,** 2411–2416.
25. Grossman, M., Raper, S. E., Kozarsky, K., Stein, E. A., Engelhardt, J. F., Muller, D., Lupien, P. J., and Wilson, J. M. (1994) *Nat. Genet.* **6,** 335–341.
26. Kobayashi, N., Fujiwara, T., Westerman, K. A., Inoue, Y., Sakaguchi, M., Noguchi, H., Miyazaki, M., Cai, J., Tanaka, N., Fox, I. J., and Leboulch, P. (2000) *Science* **287,** 1258–1262.
27. Auth, M. K., Okamoto, M., Ishida, Y., Keogh, A., Auth, S. H., Gerlach J., Enke, A., McMaster P., and Strain, A. J. (1998) *Transplant. Int.* **11**(Suppl. 1), S439–S443.
28. Burt, C. V., Wallace, L., Kelly, D. A., McMaster, P., and Strain, A. (2000) *FASEB J.* **14,** A285.
29. Gerlach, J. C. (1996) *Int. J. Artif. Organs* **19,** 645–654.
30. Mertsching, H., Frühauf, J. H., Herden, T., Haverich, A., and Bader, A. (1999) Abstracts, Annual Meeting of the European Liver Cell Network ELNET, September 17, 1999, Hamburg, Germany.
31. Runge, D. M., Runge, D., Dorko, K., Pisarov, L. A., Leckel, K., Kostrubsky, V. E., Thomas, D., Strom, S. C., and Michalopoulos, G. K. (1999) *J. Hepatol.* **30,** 365–274.
32. Runge, D., Runge, D. M., Jäger, D., Lubecki, K. A., Beer Stolz, D., Karathanasis, S., Kietzmann, T., Strom, S. C., Jungermann, K., Fleig, W. E., and Michalopoulos, G. K. (2000) *Biochem. Biophys. Res. Commun.* **269,** 46–53.
33. Guyomard, C., Rialland, L., Fremond, B., Chesné, C., and Guillouzo, A. (1996) *Toxicol. Appl. Pharmacol.* **141,** 349–356.
34. Hengstler, J. G., Utesch, D., Steinberg, P., Platt, K. L., Diener, B., Ringel, M., Swales, N., Fischer, T., Biefang, K., Gerl, M., Böttger, T., and Oesch, F. (2000) *Drug Metab. Rev.* **32,** 81–118.
35. Ferrini, J.-B., Dulic, V., Pichard-Garcia, L., and Maurel, P. (1999) *in* Short Course Manual, 7th European ISSX Meeting, pp. 109–130, Budapest.