HEPATOCYTE USERS GROUP MEETING 2004

ABSTRACT BOOK

12th-13th November 2004
El Saler (Valencia)
Spain

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The **HUG (Hepatocyte Users Group)** is an informal association of scientists from academia and industry interested in the research and application of hepatocytes and other *in vitro* liver models in biomedical research.

HUG meets every two years in a short but intensive scientific workshop to review the most recent advances in the field. The scientific sessions consist in a series of state-of the art lectures given by outstanding scientists together with invited platform presentations issued by the Scientific Committee among the submitted abstracts.

Each Meeting is focussed on one/two specific topics. The 2004 meeting is devoted to: *"HEPATOCYTE DIFFERENTIATION and GENE EXPRESSION"* and *"LONG TERM CULTURE/MARKERS OF TOXICITY"*

The 2004 meeting will take place in hotel Sidi Saler, in the vicinity of the Natural Park "La Albufera", surrounded by dunes and pine trees, and close to the *Albufera* lake. The beauty and quietness of this place (12 Km South from Valencia) offers an ideal atmosphere for scientific exchange and personal contacts.

HUG Committee:

Dr. Martin Bayliss
GlaxoSmithKline

Prof. José V. Castell
Faculty of Medicine. University of Valencia
Hospital Universitario La Fe, Valencia

Prof. Gabrielle Hawksworth
University of Aberdeen
Dep. Medicine & Therapheutics

Prof. Patrick Maurel
INSERM U632: Physiopathologie Hepatique

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SCIENTIFIC PROGRAM

Friday, 12th November

12:00 Registration and Buffet lunch in the Hotel Sidi Saler (lunch available from 12.30 pm).

14:00 General Introduction and welcome

TOPIC 1: HEPATOCYTE DIFFERENTIATION AND GENE EXPRESSION

14:15-14:50 **Prof. Nissim Benvenisty**, Professor of Genetics, The Hebrew University of Jerusalem, Israel. *"Human embryonic stem cells in medical research and their differentiation into hepatocytes"*

14:50-15:25 **Dr. Ramiro Jover** . University of Valencia, Hospital Universitario La Fe, Valencia, Spain. *"Liver-enriched transcription factors and their role in preserving the hepatic phenotype in liver cell models"*

15:25-16:00 **Prof. Patrick Maurel**, INSERM U632: Physiopathologie Hepatique. Montpellier, France. *"Long-term hepatocyte cultures: differentiation and competence"*

16:00-16:20 Tea/Coffee

16:20-16:55 **Prof. Iannis Talianidis**, Institute of Moleular Biology & Biotechnology, Herakleion, Crete, Greece. *"Dynamics of preinitiation complex assembly on the regulatory regions of differentiation-induced genes"*

16:55-17:30 **Prof. Emile Van Schaftingen**, Universite Catholique de Louvain, Belgium. *"Isolated hepatocytes as a tool to discover new short-term regulation mechanism"*.

17:30-17:45 **Prof. Tamara Vanhaecke**,Vrije Universiteit Brussel. *"Effect of HDAC inhibition on proliferation-and apoptosis-related signalling in egf-stimulated primary rat hepatocyte cultures"*.

17:45-18:00 **Ms Virginie Cerec** , Biopredic, "*HepaRG: a novel human differentiated hepatoma cell line"*.

18:00-18:15 **Ms Celia Pilar Martínez**, University of Valencia, Hospital Universitario La Fe. *"Transcriptional regulation of the human hepatic"*

18:15-18:30 **Prof. Alan Paine**, King's College:London.. "*Global analysis of hepatocyte"*.

18:30-19:30 Poster Presentations

20:30 Evening Meal

Saturday 13th November

07:30-08:30 Breakfast

TOPIC 2: LONG TERM CULTURE/MARKERS OF TOXICITY

09:00-09:35 **Prof. Paolo Bernardi**, University of Padova, Italy. *"Mitochondria as pharmacological targets for hepatoprotection"*

09:35-10:10 **Dr. Franziska Boess**, Hoffman-Laroche. Ltd, Basel, Switzerland.*"Gene expression in two hepatic cell lines, cultured primary hepatocytes, and liver slices compared with the in vivo liver gene expression in rats: possible implications for toxicogenomics and use of in vitro systems".*

10:10-10:45 **Dr. lain Uings** at GSK, UK *"Use of human hepatocytes to measure glucocorticoid receptor mediated responses to characterise novel compounds".*

10:45-11:15 Tea/Coffee

11:15-11:30 **Mr. Robert Thomas**, University of Nottingham. *"Co-Culture of Activated Primary Rat Stellate Cells with Rat or Human Hepatocytes".*

11:30-11:45 **Ms. Anne Kienhuis**, TNO Food and Nutrition / Maastricht University, *"An in vitro system for predicting toxicity by gene expression profiling".*

11:45-12:00 **Dr. Jordi Muntané**, Hospital Universitario Reina Sofía, "*PGE1-dependent nitric oxide reduces d-galactosamine-induced cell death"*

12:00-12:15 **Ms Karen McArdle**, University of Aberdeen, *"Mechanisms of cannabidiol induction of human P450s"*

12:15-12:40 **Prof. Kevin Chipman**, University of Birmingham, UK *"Down-regulation of connexins and implications for toxicity and carcinogenesis"*

12:40-13:15 **Dr. Gerry Kenna**, AstraZeneca, UK *"Use of cultured hepatocytes to support safety assessment of new pharmaceuticals"*

13:30 Lunch

15:00-15:45 Forum for collaborative projects within the 6th Framework Program. Chaired by Jose Castell.

16:00 Closing of Meeting

SESSION 1

Hepatocyte differentiation and gene expression

Friday, 12th November 2004

Session 1: Hepatocyte differentiation and gene expression

S1-C1 Human embryonic stem cells in medical research and their differentiation into hepatocytes. *Nissim Benvenisty*

S1-C2 Liver-enriched transcription factors and their role in preserving the hepatic phenotype in liver cell models. *Ramiro Jover*

S1-C3 Long-term hepatocyte cultures: differentiation and competence. *Lydiane Pichard-Garcia, Christine Biron, Martine Daujat, Sabine Gerbal-Chaloin, Edith Raulet and Patrick Maurel*

S1-C4 Dynamics of preinitiation complex assembly on the regulatory regions of differentiation-induced genes. *Antigone Kouskouti, Pantelis Hatzis and Iannis Talianidis*

S1-C5 Isolated hepatocytes as a tool to discover new short-term regulation mechanism. *Emile Van Schaftingen*

S1-O1 Effect of HDAC inhibition on proliferation-and apoptosis-related signalling. *T. Vanhaecke, P. Papeleu and V. Rogiers*

S1-O2 HepaRG: a novel human differentiated hepatoma cell line. *Cerec Virginie, Gripon Philippe, Glaise Denise, Guillouzo Christiane, Corlu Anne, Guyomard Claire, Boissier Christine*

S1-O3 Transcriptional regulation of the human hepatic *CYP3A4* by the inflammatory signal interleukin-6: molecular mechanism and transcription factors involved**.** *Celia P. Martínez-Jiménez, M. José Gómez-Lechón, José V. Castell and Ramiro Jover*

S1-O4 Global analysis of hepatocyte. *Alan J. Paine*

S1-A1 Cyt p450 1a1 and glycine conjugaton activities in rat liver spheroids. *Mingwen Ma, Jinsheng Xu and Wendy M. Purcell*

S1-A2 The effect of trichostatin a on gap junctional intercellular communication in primary cultures of rat hepatocytes. *Vinken M., Vanhaecke T., Chipman J.K. and Rogiers*

S1-A3 Effect of NAC on IGF-I splice variants in human liver following hepatectomy. *Dijk S, Fuller B, Sheth H, Goldspink G and Davidson B*

S1-A4 The european center for validation of alternative methods (ecvam) -funded pre-validation study on the response of primary human hepatocyte cultures to model cyp inducers. *Lysiane Richert, Catherine Viollon-Abadie, Alexandre Bonet, Bruno Heyd, Georges Mantion, Eliane Alexandre, Daniel Jaeck Samantha Orr, Rakhee Patel, Shaun Kingston, Clare Pattenden, Ashley Dennison, Edward leCuyse*

S1-A5 Organic anion transporting polypeptide levels can be altered by known microsomal enzyme inducers. *D.E.Cowie, R.J.Weaver, G.M.Hawksworth*

HUMAN EMBRYONIC STEM CELLS IN MEDICAL RESEARCH AND THEIR DIFFERENTIATION INTO HEPATOCYTES

Nissim Benvenisty

Department of Genetics, The Hebrew University, Jerusalem, Israel.

Human embryonic stem cells (HESCs) are pluripotent cells that can serve as a cell source for transplantation medicine, and as a tool to study human embryogenesis. We have shown that differentiate of HESCs can be induced in vitro, through formation of structures called embryoid bodies. The human embryoid bodies comprise of cells from the three embryonic germ layers, and some of these bodies express myocardial markers and are pulsating in rhythm. In addition, differentiation of HESCs may be directed by growth factors. Thus, a dozen of different cell types have been shown to differentiate in response to the presence of various secreted molecules. Moreover, these cells may be genetically modified in culture and thus specific differentiated cell types can be labeled and sorted at different stages of their differentiation. This analysis sets the stage for directing differentiation of HESCs in culture, and isolation of specific cell types for cellular transplantation. Recently, we have investigated the potential of HESCs to differentiate into hepatic cells. We have characterized the expression level of liver-enriched genes in undifferentiated and differentiated HESCs by DNA microarrays. Our analysis revealed a subset of fetal hepatic enriched genes that are expressed in HESCs upon differentiation into embryoid bodies. In order to isolate the hepatic-like cells, we introduced a reporter gene regulated by a hepatocyte-specific promoter into HESCs. We isolated clones of HESCs that express enhanced green fluorescent protein upon in vitro differentiation. Through immunostaining, we showed that most of these cells express albumin, while some cells still express the earlier expressed protein alpha-fetoprotein. Using fluorescence activated cell sorter, we were able to sort out the fluorescent differentiated cells and expand them for a few more weeks. As observed during normal embryonic development, we showed that in teratomas, the hepatic-like endodermal cells develop next to cardiac mesodermal cells. In order to examine the secreted factors involved in the induction of hepatic differentiation, HESCs were grown in the presence of various growth factors, demonstrating the potential involvement of acidic fibroblast growth factor in the differentiation. In conclusion, given certain growth conditions and genetic manipulation, we can now differentiate and isolate hepatic-like cells from HESCs.

LIVER-ENRICHED TRANSCRIPTION FACTORS AND THEIR ROLE IN PRESERVING THE HEPATIC PHENOTYPE IN LIVER CELL MODELS

Ramiro Jover

Hospital Universitario "La Fe", Valencia; Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad de Valencia, Spain.

The liver performs essential functions in the body by expressing hepatocyte-specific genes encoding plasma proteins, clotting factors and enzymes involved in detoxification, gluconeogenesis, glycogen synthesis, and glucose, fat, and cholesterol metabolism. Functional analysis of numerous hepatocyte-specific DNA regulatory regions reveals that they are composed of multiple cis-acting DNA sequences that bind different families of liver-enriched transcription factors (LETF) named hepatocyte nuclear factor (HNF) families and CCAAT/enhancer binding protein (C/EBP) family. The results of gene reporter analysis using DNA regulatory regions containing targeted mutations in LETF binding sites suggested that hepatocyte-specific gene transcription requires simultaneous binding of multiple, distinct LETF to the gene regulatory region providing synergistic transcriptional activation. The requirement for combinatorial protein interactions between multiple LETF to achieve synergistic stimulation of hepatocyte-specific genes plays an important role in maintaining liver-specific gene expression. Furthermore, maintenance of hepatocyte-specific expression of any of the LETF, in turn, involves cross-regulation by other LETF transcription factors.

Cultured primary human hepatocytes are the model of choice to study specific liver functions such as drug metabolism and detoxification. Only primary cultures can produce the metabolic profile of a drug similar to that found *in vivo* and can respond to inducers. However, because of their limited accessibility, alternatives to replace human hepatocytes are currently being explored. Unfortunately, immortalized hepatocytes or hepatoma cells do not preserve the desirable phenotypic characteristics to replace primary cultures in drug metabolisms studies. We investigated why human hepatoma cells do not express drug-metabolizing CYP genes and found that the levels of several key transcription factors clearly differ from those found in hepatocytes. It was then conceivable that re-expression of one (or more) of these transcription factors could lead to an efficient transcription of CYP genes. The feasibility of this hypothesis was demonstrated by genetic engineering of Hep G2 cells with liver-enriched transcription factors followed by the analysis of the expression of the most relevant human CYPs. HNF3g resulted to be a potent transactivator of CYPs of the 2C subfamily, while C/EBPa showed an effective activation on CYP3A genes, as demonstrated by overexpression experiments with adenoviral vectors. Moreover, C/EBPa and HNF3g cooperatively regulated CYP3A4 expression by a mechanism likely involving chromatin remodelling. Studies using adenovirus-mediated antisense targeting have identified HNF4 as an important direct transactivator of major drug-metabolising CYP genes. The broad dependence of CYPs on HNF4 leads us to consider this factor as a global regulator supporting CYP expression in human liver. In hepatoma cells however, total HNF4 is present at levels as high as in hepatocytes, yet its activity on targets promoters seems to be very low or even absent. Our experimental evidence suggests that the low level of expression of the Steroid Receptor Coactivators (SRCs) could be a major reason for the lack of function of HNF4 in hepatoma cells. In summary, tailored re-expression of missing activators and co-activators in hepatoma cells can lead to a significant re-expression of relevant CYPs, opening a new experimental strategy to metabolically upgrade human hepatoma cells for human drug metabolism and toxicity studies.

LONG-TERM HEPATOCYTE CULTURES: DIFFERENTIATION AND COMPETENCE

Lydiane Pichard-Garcia, Christine Biron, Martine Daujat, Sabine Gerbal-Chaloin, Edith Raulet and Patrick Maurel

INSERM U632, University Montpellier-1 EA3768, Hepatic Physiopathology, 1919 Route de Mende, F-34293 Montpellier

We have developped experimental conditions for long-term human hepatocyte cultures (LTHC). Several liver phenotypic markers have been evaluated to document the degree of differentiation of the cells. We have shown that the production of plasma proteins such as albumin and alpha 1-antitrypsin, or lipoproteins (Lpa, ApoB100), or the production of urea is maintained at a constant or even increasing level for at least one month (Chem Biol Inter 1997). The cells respond to cytokines (notably Il-1 and interferon alpha) in terms of activation of the signal transmission cascades (Hepatology 2004 ; J Virol 2002). Liver-enriched transcription factors such as C/EBP and HNF4 are expressed and functional in these LTHC (J Hepatol 2001). As liver plays a major role in haemostasis, we recently investigated the production of haemostasis proteins by our LTHC. Analysis of cell extracellular medium by immunological methods and coagulant activity measurement revealed the presence of factors II, V, VII, VIII, PIVKA-II, fibrinogen and antithrombin. Addition of vitamin K1 to the culture medium resulted in a significant increase of factors II and VII and a reciprocal decrease of PIVKA-II ; addition of von Willebrand factor resulted in a drastic increase in factor VIII (Brit J Haematol 2004). For many years we have been using these cultures to investigate the expression and regulation of CYP genes. Our data show that CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A7 are expressed and enzymatically active in LTHC for at least one month, provided appropriate inducers or cocktail of inducers including dioxin, phenobarbital and rifampicin are used (Methods Enzymol 2002). We have used these LTHC to study the regulation of the AhR by omeprazole (OM) and omeprazole sulphide (OMS). Using a battery of tests and hepatocarcinoma cell lines (Hepa-1 and HepG2), we showed that OMS is an antagonist of the human and mouse AhR. Upon binding, OMS blocks the receptor nuclear translocation and DNA binding. However, when OMS was tested in freshly plated human hepatocytes it behaved as an AhR agonist and induced CYP1A1 and 1A2, as shown previously for OM (J Biol Chem 1997). In fact, modulations of drug metabolism activity of these cultures through the use of inhibitor (ketoconazole) and inducer (rifampicin) led to parallel modulations of the properties of OMS which changed from antagonist to agonist of the AhR and *vice versa*. Thus, drug metabolising enzymes (likely to convert OMS to OM) play a critical role in controlling the functional interactions between OMS and the AhR. We conclude that erroneous conclusions may be drawn from experiments carried out with hepatoma cell lines.

MOLECULAR MECHANISM OF PREINITIATION COMPLEX FORMATION ON LIVER SPECIFIC GENES AND THE MAINTENANCE OF ACTIVE CHROMATIN STATE DURING CELL DIVISION

Antigone Kouskouti, Pantelis Hatzis and Iannis Talianidis

Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology Hellas, 1527 Vassilika Vouton, 711 10, Herakleion, Crete, GREECE

Dynamic changes in the structure of chromatin at gene regulatory regions have been implicated in the processes of gene repression and activation, as well as in the propagation of epigenetic information

Active genes are characterized by specific histone modification marks, including acetylation and H3-K4 methylation. Analysis of the recruitment of preinitiation complex components on promoters upon the initial activation of genes during cellular differentiation revealed a step-wise mechanism, in which histone modifications play a crucial role in the formation of active preinitiation complex. Following activation, most genes remain active through many cell divisions, thus establishing a given array of stably expressed genes characteristic for a given cell type. The potential role of histone modifications on the epigenetic maintenance of the active chromatin configuration was addressed by studying the pattern of a wide range of histone modifications at the promoter and the coding regions of constitutively active hepatic genes. We observed a surprisingly stable pattern of H3 and H4 acetylation along with H3-K79 and H3-K4 methylation on the promoters and the 5' portion of the coding regions in G1 phase-enriched, mitotically arrested and a-amanitin-blocked cells. This pattern correlated with gene-specific and transcription-dependent translocation of histone acetyltransferases and chromatin-remodeling factors into the coding regions, which resulted in a localized pattern of histone modifications and structural alterations of nucleosomes downstream of the promoters.

The results provide new insights into the molecular events that lead to the creation of a wider open chromatin domain around the promoters of active genes. Given the stability and the specific distribution of histone modifications within these domains, we propose that they may serve as molecular memory marks for the transmission of the active chromatin state during cell divisions.

ISOLATED HEPATOCYTES AS A TOOL TO DISCOVER NEW SHORT-TERM REGULATION MECHANISMS

Emile Van Schaftingen

Laboratory of Physiological Chemistry, Christian de Duve Institute of Cellular Pathology and Université catholique de Louvain, Brussels Belgium

The purpose of the present talk is to show the advantages of isolated hepatocytes as a model to analyse problems of metabolic regulation. Two different examples of studies that led to the discovery of new regulation mechanisms will be used to illustrate this : the discoveries of fructose 2,6-bisphosphate and of glucokinase regulatory protein.

Fructose 2,6-bisphosphate was identified as a result of studies aimed at understanding the mechanism by which glucagon stimulates glucose production in liver. Metabolic flux measurements and metabolite assays, performed to a large extent on isolated hepatocytes, have indicated that glucagon acts at two level on glucose formation : between pyruvate and phosphoenolpyruvate on the one hand, and between fructose 1,6-bisphosphate and fructose 6-phosphate on the other hand. The use of isolated hepatocytes was also instrumental in identifying that pyruvate kinase is regulated by covalent modification, catalysed by cAMP-dependent protein kinase. It led also to the finding that the kinetic properties of phosphofructokinase were modified in crude extracts from hepatocytes treated with glucagon as compared to crude extracts of control cells. This modification disappeared after gel-filtration of the extracts and could be attributed to a change in the concentration of a low-molecular-weight stimulator of phosphofructokinase, which was subsequently identified as fructose 2,6-bisphosphate. Later studies on isolated hepatocytes confirmed the role of this molecule as an important physiological effector of phosphofructokinase and fructose 1,6-bisphosphatase.

Glucokinase-regulatory protein (GK-RP) was identified in a study aimed at understanding the effect of fructose on glucose phosphorylation in the liver. This effect, was first identified in isolated hepatocytes by measuring glucose phosphorylation through the formation of tritiated water from [2-^{3}H] glucose. Specificity studies indicated that the effect was also observed with D-glyceraldehyde, which may condense enzymatically with dihydroxyacetone-phosphate to form fructose-1-phosphate. These studies, in which hepatocytes played a crucial role, led to the concept that fructose 1-phosphate is a positive effector of glucokinase. Further work showed that this effect of fructose-1-phosphate requires a regulatory protein that allosterically controls glucokinase. Isolated hepatocytes played also a major in the identification by L. Agius that glucose and fructose cause translocation of glucokinase from the nucleus to the cytosol, an effect in which the glucokinase/GK-RP interaction plays a major role.

Would these discoveries have been made without the isolated hepatocyte model? This is far from warranted. Had fructose 2,6-bisphophate not been discovered in 1980, the sequence of the various forms of PFK2-FBPase2, which catalyse the synthesis and degradation of this effector, would certainly be known as a result of the sequencing of genomes, but they would probably still be ‘proteins with unknown function’.

Their low homology with adenylate kinase and phosphoglycerate mutase would certainly be more a puzzle than a help to identify their catalytic function, most particularly since PFK2-FBPase2's (unlike their distant homologs) have extremely low turnover numbers and catalyze the synthesis (and degradation) of a chemically labile compound.

Freshly isolated hepatocytes have several advantages compared to more complex models (*in vivo*, perfused liver) : ease of preparation, possibility of comparing a number of different experimental conditions in a single experiment, lack of effect of hormones and vasoactive agents secreted by other cells. Compared to cultured hepatocytes, they have the advantages of being available in larger amounts (which may be critical when biochemist want to decipher a new regulatory mechanism) and of being well-differentiated and with the normal complement of hepatic enzymes. Unlike cell-free systems, the intracellular milieu has a close to physiological ionic composition and pH, which is very important for many regulatory processes that can take place only in a narrow window of experimental conditions. Freshly isolated hepatocytes therefore still have a place to decipher new regulatory mechanisms.

EFFECT OF HDAC INHIBITION ON PROLIFERATION- AND APOPTOSIS-RELATED SIGNALING IN EGF-STIMULATED PRIMARY RAT HEPATOCYTE CULTURES

T. Vanhaecke, P. Papeleu and V. Rogiers

Department of Toxicology, Vrije Universiteit Brussel (VUB), Laarbeeklaan 103, 1090 Brussels, Belgium

The level of histone acetylation, determined by the balance between histone acetyltransferase activity and histone deacetylase (HDAC) activity, is crucial for gene transcription. As such, the HDAC inhibitor Trichostatin A (TSA) has been shown to interfere with proliferation, differentiation and apoptosis in tumor cells, including hepatoma cell lines. Here, we investigated the effects of TSA on cell cycle progression and survival in EGF-stimulated cultures of primary rat hepatocytes. It was found that 1µM TSA completely abolished DNA synthesis at 60h and this was accompanied by induced histone H4 acetylation. In addition, in the presence of TSA, the S-phase marker cdk1 was absent, whereas all G1 (cyclins D1 and E, cdk4) as well as G1/S (cyclin A) transition markers were present. However, the expression of p21 was not altered upon TSA treatment. In contrast to hepatoma cells, TSA also reduced spontaneous apoptosis as evidenced by a reduction of procaspase-3 cleavage, decreased expression of Bid and increased expression of BclxL. Moreover, with TSA, increased albumin secretion by the hepatocytes was observed In conclusion, our results show that next to the induction of an early, p21-independent S-phase arrest, TSA also potentiates the anti-apoptotic effect of EGF in primary hepatocytes and positively affects hepatocyte functioning.

S1-O2

HEPARG: A NOVEL HUMAN DIFFERENTIATED HEPATOMA CELL LINE EXHIBITING HIGH LEVELS OF INDUCIBLE CYP450S AND GSTS ENZYMES USEFUL FOR PHARMACOLOGICAL AND TOXICOLOGICAL APPLICATIONS

[1]Cerec Virginie, Gripon Philippe, Glaise Denise, Guillouzo Christiane, Corlu Anne [2]Guyomard Claire, Boissier Christine

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HepaRG, a novel human hepatoma cell line, derived from a differentiated hepatocarcinoma is unique in: i) its susceptibility to hepatitis B virus infection, ii) its tremendous plasticity switching in vitro from an undifferentiated state characterized by active proliferation, to a highly differentiated state, morphologically close to human hepatocytes, iii) its bipotent biliary and hepatocytic differentiation pathway capacities. At the ultimate stage of the hepatocytic differentiation process, HepaRG hepatocyte-like cells exhibit specific hepatocyte functions such as the synthesis of liver plasma proteins, sugar metabolism enzymes and a large number of phase I and phase II drug metabolism enzyme activities belonging to CYPs and GSTs families. Inducibility of CYP1A and CYP3A by respectively 3-methylcholantrene and rifampicin has also been demonstrated. Levels of basal and induced CYP450s are found in the range of the ones obtained in normal human hepatocytes. For all these reasons, HepaRG is a new powerful tool in the fields of virology, parasitology, pharmacology and toxicology.

S1-O3

TRANSCRIPTIONAL REGULATION OF THE HUMAN HEPATIC *CYP3A4* BY THE INFLAMMATORY SIGNAL INTERLEUKIN-6: MOLECULAR MECHANISM AND TRANSCRIPTION FACTORS INVOLVED.

Celia P. Martínez-Jiménez, M. José Gómez-Lechón, José V. Castell and Ramiro Jover

Unidad de Hepatología Experimental, Centro de Investigación, Hospital Universitario "La Fe", Valencia; and Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad de Valencia, Spain.

Drug-metabolizing cytochromes P450 (CYPs) are down-regulated during inflammation in the liver**.** *In vitro* studies have shown that pro-inflammatory cytokines are largely responsible for this CYP repression. However, the signalling pathway(s), the activated transcription factor(s) and their target site(s) in the CYP genes remain to be properly identified. We have investigated the negative regulation of CYP3A4 (the major drug-metabolizing human CYP) by interleukin-6 (IL-6) (the principal regulator of the hepatic acute-phase response). Our results showed that *CYP3A4* down-regulation by IL-6 requires activation of the glycoprotein receptor gp130; but it does not proceed by the JAK/STAT pathway nor by activation of ERK1/2 or p38 kinases. Noteworthy, IL-6 caused a moderated induction in the mRNA of the transcription factor C/EBPβ and a marked increase in the translation of C/EBPβ-LIP, a 20 kDa C/EBPβ isoform lacking transactivation domain. Adenovirus-mediated expression of C/EBPβ-LIP caused a dose dependent repression of *CYP3A4*, whereas over expression of C/EBPβ-LAP (35 kDa) caused a significant induction. Analysis of *CYP3A4* 5'-flanking region demonstrated the existence of a 288 bp sequence at -5.95 kb, which showed maximal response to C/EBPβ-LAP (~23 fold increase in HepG2 cells). Co-expression of LAP with increasing amounts of LIP reduced this activating effect by 70%. Site-directed deletions of predicted C/EBPβ binding sites demonstrated the presence of three functional C/EBPβ responsive motifs within this distal flanking region. Further experiments using chromatin immunoprecipitation proved the binding of endogenous C/EBPβ to the -5.95 kb enhancer of the *CYP3A4* gene in human hepatocytes, and, when recombinant LAP and LIP were expressed, increased binding to this region was also observed. Taken together, our results uncover a novel mechanism for *CYP3A4* down regulation by pro-inflammatory cytokines in human hepatocytes. Our experimental evidence supports the idea that IL-6 down-regulates CYP3A4 through translational induction of C/EBPβ-LIP, which competes with and antagonizes constitutive C/EBP transactivators. Competition, binding and repression by C/EBPβ-LIP occur through a new distal enhancer site at -5.95 kb in the *CYP3A4* gene. From a clinical point of view, these findings could be of relevance because many physiopathological conditions causing LIP up-regulation could contribute to the *CYP3A4* inter-individual variability, which represents a basis for drug interactions and toxicities.

GLOBAL ANALYSIS OF HEPATOCYTE GENE EXPRESSION AND TARGETS FOR RE-ESTABLISHING THEIR DIFFERENCIATED PHENOTYPE IN CULTURE.

Alan J. Paine

Pharmaceutical Sciences Research Division, Franklin-Wilkins Building, King's College, London, U.K.

Analysis of the expression of over 8000 rat genes by DNA micro-array technology in rat hepatocytes during the first 72 hours of culture detects about 2610 transcripts. Of these, 10% increase or decrease by more than 2 fold during the first 4 hours of culture rising, 12 hours post plating, to 25%. Between 24 and 72 hours of culture these pronounced, initial, changes in hepatocyte gene expression remain relatively constant affecting about 30% of the total transcripts detected. However, cluster analysis into self organising maps indicates at least 64 patterns of response and hence a major reprogramming of liver cell gene expression as hepatocytes recover from their isolation and adapt to culture. Therefore, given that thousands of transcripts are changing over this 72 hour time frame only general trends relevant to the application of hepatocyte cultures to the meeting theme of drug metabolism/toxicity and hepatocyte differentiation will be considered.

In this respect, one of the most striking and hither too unreported changes in hepatocyte gene expression is a 10 fold increase by 4 hours of culture in transcripts encoding "acute phase response" proteins. These transcripts are increased 20 fold by 12 hours of culture and remain at high levels for 48 hours post plating indicating that hepatocytes are undergoing a prolonged and major inflammatory response that is well known to lead to a loss of differentiated phenotype in vivo (e.g. suppression of cytochrome P450 mediated drug metabolism). This hypothesis is commensurate with the finding that the transcription factor NF-?B, a powerful mediator of inflammation, is activated to a DNA binding species just 15 minutes into the well established hepatocyte isolation procedure. Furthermore, signalling pathways (MAP kinases) that lead to activation of NF-?B and other transcription factors are activated within minutes of commencing hepatocyte isolation suggesting that subsequent reprogramming of hepatocyte gene expression is initiated during the conventional isolation procedure. In view of the speed of these responses they are unlikely amenable to pharmacological intervention so we are attempting to evaluate the role of MAP kinases and hence hopefully reprogram hepatocyte de-differentiation (improving their utility in drug metabolism and toxicity studies etc.) by knocking down specific signalling pathways in the liver donor, through the new technique of RNA interference, prior to hepatocyte isolation and culture.

S1-A1

CYT P450 1A1 AND GLYCINE CONJUGATON ACTIVITIES IN RAT LIVER SPHEROIDS

Mingwen Ma, Jinsheng Xu and Wendy M. Purcell

Centre for Research in Biomedicine, Faculty of Applied Sciences, University of the West of England, Bristol, UK.

Liver is an important organ in drug metabolism and liver *in vitro* models have been widely used in drug metabolism studies. However, cell isolation procedures can adversely affect hepatocytes and cause functionality to deteriorate during subsequent monolayer cultures (1). Liver spheroid culture has been shown to be superior to hepatocyte monolayer culture as an *in vitro* model. In particular, key functionality is recovered in spheroid culture (2-3). This study investigated the changes in phase I and II metabolism in liver spheroids. Spheroids were prepared from primary hepatocytes isolated from rat liver by a gyrotatory method. P450 1A1 (phase I) and glycine conjugation (phase II) activities were evaluated by using specific substrates, ethoxyresorufin and benzoic acid respectively, over 21 days. Results show that P450 1A1 activity was just detectable in the first two days and recovered and was maintained in mature spheroids (> 5 days). Cell isolation significantly increased glycine conjugation activity, which decreased to a lower level within 3 days and was maintained over the 21-day period of observation. It is concluded that drug metabolic activity in hepatocytes undergoes changes during liver spheroid formation and maintenance. Mature liver spheroids maintain higher levels of P450 1A1 metabolic activity and therefore are suitable for drug metabolism studies. Liver spheroids from days 1 to 9 appear to be suitable for glycine conjugation studies.

References

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S1-A2

THE EFFECT OF TRICHOSTATIN A ON GAP JUNCTIONAL INTERCELLULAR COMMUNICATION IN PRIMARY CULTURES OF RAT HEPATOCYTES

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Trichostatin A (TSA), a histone deacetylase inhibitor, is known to exert major effects on the cellular homeostatic balance. As this equilibrium is partly controlled by gap junctions, being composed of connexin (Cx) proteins, the question was raised whether TSA might affect the expression of Cx proteins in primary cultures of rat hepatocytes. Therefore, rat hepatocytes were isolated by collagenase perfusion and the isolated cells were subsequently cultivated for 7 days in1 μM TSA-supplemented medium. By using immunoblotting and -histochemistry, increased expressions of Cx32 and Cx43, and decreased expression of Cx26 were observed. Overall, enhanced gap junction activity was observed, by using the scrape loading/dye transfer assay. This observation was associated with an increased albumin secretory activity, measured by ELISA. From these results, two major conclusions can be drawn: (i) TSA promotes gap junctional intercellular communication in primary cultures of rat hepatocytes, (ii) Cx proteins fulfil differential roles in the control of hepatic homeostasis and TSA is an interesting tool to further explore this notion.

S1-A3

EFFECT OF NAC ON IGF-I SPLICE VARIANTS IN HUMAN LIVER FOLLOWING HEPATECTOMY

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Liver ischemia-reperfusion (I/R) is a major course of morbidity and mortality in patients undergoing liver resections for cancer. The development of an effective medical treatment to counter this is desirable. N-acetyl-cysteine (NAC) has shown efficacy in modulating liver I/R. To understand the molecular pathways activated by NAC in liver tissue, tru-cut biopsies were taken from patients undergoing liver resections who received either NAC or placebo. In each patient a biopsy was taken before the start and after completion of the liver resection and compared for expression of Insulin-like growth factor I (IGF-I) splice variants (Ea and Ec), using Real-time PCR and specific primers. The Ea splice variant (full-length IGF-I) was detectable in both NAC and control group, but no difference between pre- and post surgery biopsy was seen in either group. Pre-surgery biopsy levels of the Ec splice variant was $6.4.10^{-8} \pm 3.9.10^{-8}$ (SD) ng in the placebo group and $5.6.10^{-8} \pm 6.2.10^{-8}$ ng in the NAC group. In the post-surgery biopsies the Ec splice variant was undetectable in the placebo group and $1.4.10^{-7} \pm 1.0.10^{-7}$ ng in the NAC group. The Ec splice variant may stimulate liver regeneration or prevent apoptosis following liver I/R.

THE EUROPEAN CENTER FOR VALIDATION OF ALTERNATIVE METHODS (ECVAM) -FUNDED PRE-VALIDATION STUDY ON THE RESPONSE OF PRIMARY HUMAN HEPATOCYTE CULTURES TO MODEL CYP INDUCERS

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The purpose of a first stage of this pre-validation study was to set criteria for optimization and harmonization of hepatocyte (HC) isolation from human tissue among laboratories to establish a routine procedure. This was achieved by combining and/or comparing the data independently generated by the four participating laboratories [(1,2)]. In a second stage of this pre-validation study, each of the four participants performed a minimum of 5 perfusions according to the retained criteria: biopsy sizes between 50-100g, cannulation with 2-4 cannulae, and digestion with collagenase-containing medium at a flow rate of 25 ml/cannula for 20 minutes, and cut surface being glued in order to reform Glisson's capsule. The three same batches of powdered Sigma collagenase (400-600 U/mg protein) tested by the participants gave a similar yield of viable HC/g liver (about 12.05 ± 1.72 $x10^6$), with no significant inter-laboratory difference. Slight modifications in the perfusion buffers (buffer A: HBSS+0.5% BSA+ 0.5mM EGTA; buffer B: PBS + 0.5mM EGTA) did not significantly affect yield of viable HC/g liver (9.17 ± 1.69 $x10^6$: buffer A; 12.18 ± 2 $x10^6$: buffer B). After Percoll purification, an average yield of 7.21 ± 1.21 $x10^6$ viable HC/g liver with a viability of preparations ranging between 70 and 80% was obtained when using powdered collagenase, with no significant inter-laboratory difference. When using the InVitrogen Hepatocyte Digest Medium the yield of viable HC/g liver was significantly lower (3.87 ± 1.07 $x10^6$), however the viability of the preparations was very high (89.46 % ± 2), thus no Percoll purification was requested before HC plating. In all cases, the plating efficiency was of about 80%, as assessed by microscopic observation, without any significant inter-laboratory difference. Each laboratory performed three to five independent human HC isolations and cultures in which the response to a 72 hour treatment period with either Naphthoflavone (2, 10, 50 μM), Rifampicine (0.1, 1, 10 μM) or Phenobarbital (50, 250, 1000 μM) was assessed in terms of cytochrome P450 (CYP)-dependent enzyme activities and protein content. An average of 68 μg microsomal protein was obtained /10^6 viable plated human hepatocytes and harvested after the 72-hour treatment period (5-6 days following seeding), with no significant inter-laboratory difference, confirming equivalent seeding and plating efficiency between laboratories and no significant treatment-related effect on total cellular microsomal proteins. A concentration-dependent response of CYPs to specific inducers was observed in all cultures. The extent of the response observed was clearly donor-dependent.

[1] Richert et al., Liver International 2004, 24 : 371-378
[2] LeCluyse et al., Methods in Molecular Biology 2004

ORGANIC ANION TRANSPORTING POLYPEPTIDE LEVELS CAN BE ALTERED BY KNOWN MICROSOMAL ENZYME INDUCERS.

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The organic anion transporting polypeptides (Oatps) constitute a major class of transporters at the basolateral membrane of hepatocytes. Nuclear hormone receptors such as PXR and CAR are implicated in the transcriptional regulation of cytochrome P450s and more recently have been implicated in the regulation of Oatps. Hepatic microsomal enzymes and transporters form part of a co-ordinately regulated response to drugs.
Male Sprague-Dawley rats were dosed intraperitoneally with PXR ligands (PCN & dexamethasone) and the CAR ligand, TCPOBOP, and the CAR activator phenobarbitone. A dose-dependent increase in the levels of Oatp1a4 protein was observed, indicating that both PXR and CAR are involved in the regulation of Oatp1a4. The optimal dose of dexamethasone was 150mg/kg, while 120mg/kg phenobarbitone, 40mg/kg PCN and 1mg/kg TCPOBOP resulted in a similar magnitude of increase. The dosages used above did not alter the levels of Oatp1a1 suggesting that nuclear receptors are not involved its regulation.
Rat hepatocytes were cultured in a 'Matrigel' sandwich with William's E medium supplemented by 100nM dexamethasone and 1.2% v/v insulin transferrin sodium - selenite. The function of the nuclear receptors, PXR and CAR, in the system was assessed by the response of CYP3A23 (PXR) and CYP2B1/2 (CAR) to the P450 enzyme inducers, dexamethasone (10μM) and phenobarbitone (500μM). Both compounds showed induction of CYP3A23 and CYP2B1/2 respectively. The effect of nuclear receptor ligands on levels of Oatp1a4 *in vitro* was ascertained. 1μM TCPOBOP, 10μM dexamethasone and 25μM PCN increased the levels of Oatp1a4, while 500μM phenobarbitone, 10μM PCN, 10 & 25μM RU486 had no effect on Oatp1a4 protein levels. These results were found to not to be reproducible when culturing cells on a single layer of Matrigel.

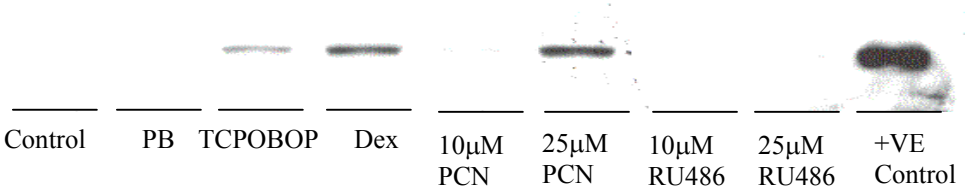

Figure 1: The *in vitro* expression of Oatp1a4 in response to known microsomal enzyme inducers.

These results indicate that under Matrigel sandwich cell culture conditions, both PXR and CAR ligands can increase the levels of Oapt1a4 protein. A Matrigel sandwich cell culture system has the potential to be used for studying the factors involved in the regulation of Oatps.

SESSION 2

Long term culture/markers of toxicity

Saturday, 13th November 2004

Session 2: Long Term Culture/Markers of Toxicity

S2-C6 Mitochondria as pharmacological targets for hepatoprotection. *Luca Nicolosi, Maria Eugenia Soriano and Paolo Bernardi*

S2-C7 Gene expression in two hepatic cell lines, cultured primary hepatocytes and liver slices compared to the in vivo liver gene expression in rats: possible implications for toxicogenomics use of in vitro systems. *Franziska Boess, Stefan Ruepp, Th. Weiser and L. Suter*

S2-C8 Use of human hepatocytes to measure glucocorticoid receptor mediated responses for characterisation of novel anti-inflammatory compounds. *Iain Uings and Stuart N Farrow*

S2-O5 Co-culture of activated primary rat stellate cells with rat or human hepatocytes on poly-(dl-lactic acid) to rapidly produce functional cell spheroids. *Thomas, Robert J; Bhandari, Rena; Bennett, Andrew; Fry, Jeff; Shakesheff, Kevin M*

S2-O6 An in vitro system for predicting toxicity by gene expression profiling. *Kienhuis AS, Wortelboer HM, van Delft JHM, Kleinjans JCS, Stierum RH.*

S2-O7 PGE1-dependent nitric oxide reduces d-galactosamine-induced cell death through attenuation of nf-kb activation and inos expression: in vivo and in vitro studies. *Jordi Muntané, Francisco Javier Rodríguez, Ana Quintero, Amira Mohamed Kamal el-Said, Emilio Siendones, Dalia Fouad, José Luis Montero, Manuel de la Mata*

S2-O8 Mechanisms of cannabidiol induction of human P450s: utilisation of hepg2-based reporter assay and primary human hepatocytes. *Karen E McArdle, Mathew C Wright, Gabrielle M Hawksworth*

S2-C9 Down-regulation of connexins and implications for toxicity and carcinogenesis. *Chipman JK, Mally A and Edwards G O*

S2-C10 Use of cultured hepatocytes to support safety assessment of new pharmaceuticals. *Gerry Kenna, Jane Hopwood, Julie Eakins and Helen Powell*

S2-A6 Human liver spheroid culture: morphology and functionality. *Jinsheng Xu, Mingwen Ma and Wendy M. Purcell*

S2-A7 Long-term 3D-culture of liver explants in relative microgravity: new perspectives and possible applications. *Nathalie Steimberg, Francesca Rovetta, Jennifer Boniotti and Giovanna Mazzoleni*

S2-A8 Influence of different parameters on cryopreserved hepatocytes. *E.Gottwald, B. Lahni, A. Katsen-Globa, H. Zimmermann, K.-F. Weibezahn*

S2-A9 Reaction phenotyping using both drug depletion and metabolite formation. *Ruben de Kanter, Elena Melara and Mario Monshouwer*

S2-A10 Plasma-based surface modification for tissue engineering purposes: the effect on hepatocyte attachment and functionality. *C. Dehili, P. Lee, M. Alexander, K. Shakesheff*

S2-A11 Optimization of the technique of hypothermic storage of equine isolated hepatocytes. *Bakala Adam, Karlik Wojciech, Wiechetek Maria, Borzecka Katarzyna, Chlopecka Magdalena, Dziekan Natalia*

S2-A12 Introducing the measurements for biotechnology programme 2004-07. *Keith Anderson, Helen Parkes , Nathan Griffiths , Peter Evans*

S2-A13 Liver grafts preseved in celsior solution as source of hepatocytes for drug metabolism studies: comparison with surgical liver biopsies**.** *M. Teresa Donato, Alfonso Serralta, Nuria Jiménez, Gabriela Pérez, José V. Castell, José Mir and M. José Gómez-Lechón*

S2-A14 Testosterone Hydroxylation by Human Hepatocytes with and without Cryopreservation. *Sabine Kafert-Kasting[1], Marc Barthold[1], Gerhard Friedrich[2], Thorsten Rose[2], Alexander Wawkuschewski[2], Lubomir Arseniev[1,3], Klaus Rissler[4]*

S2-A15 Utility of long-term cultured human hepatocytes as an *in vitro* model for cytochrome p450 induction. *Georgina Meneses-Lorente[(1)], Christine Pattison[(1)], Maria Dominguez[(1)], Robert Heavens[(1)], Bindi Sohal[(1)], Gwnéaëlle Caroff[(2)], Claire Guyomard[(2)]*

MITOCHONDRIA AS PHARMACOLOGICAL TARGETS FOR HEPATOPROTECTION

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We are investigating the role of mitochondria in the pathogenesis of experimental fulminant hepatitis and as potential targets for pharmacological intervention in rats and CD10 mice. Stimulation of the Tumor Necrosis Factor (TNF) alpha receptor with E. Coli lypopolysaccharide (LPS) plus D-Galactosamine (D-GalN) or of the Fas (CD95, APO-1) receptor with the JO2 antibody caused acute hepatotoxicity and death of treated CD10 mice within about 12 hours. Death was preceded by the cleavage of caspases 8 and 3 in liver homogenates, and by the appearance of large numbers of TUNEL-positive hepatocytes in liver sections. We determined the optimal dose of cyclosporin A (CsA) needed to inhibit the permeability transition pore (PTP) in vivo through a sensitive assay recently developed in our laboratory [Soriano et al. (2004) J. Biol. Chem. 279, 36803]. At a dose of 5-10 mg x kg-1 body weight CsA inhibited the PTP ex vivo; protected from acute hepatotoxicity, as judged from inhibition of caspase 3 cleavage; and greatly reduced the number of TUNEL-positive hepatocytes. Since caspase 8 cleavage was not affected, these findings demonstrate that CsA does not inhibit activation of the death receptors (TNFalpha and Fas) but rather acts downstream of caspase 8 by inhibiting the mitochondrial proapoptotic pathway. Thus, PTP-dependent triggering of the mitochondrial apoptotic pathway plays an important role in the pathogenesis of liver damage caused by stimulation of the TNFalpha and Fas receptors. Our initial experiments indicate that treatment with CsA also resulted in the long-term survival of mice, suggesting a potential use for CsA in the treatment of fulminant hepatitis and establishing an experimental paradigm that can be exploited for the development and testing of hepatoprotective drugs.

S2-C7

GENE EXPRESSION IN TWO HEPATIC CELL LINES, CULTURED PRIMARY HEPATOCYTES AND LIVER SLICES COMPARED TO THE IN VIVO LIVER GENE EXPRESSION IN RATS: POSSIBLE IMPLICATIONS FOR TOXICOGENOMICS USE OF IN VITRO SYSTEMS.

Franziska Boess, Stefan Ruepp, Th. Weiser and L. Suter

In the past several years, microarray technology has quickly emerged, allowing genome-wide analysis of gene expression at the RNA level. In the field of Toxicogenomics this technology could help to identify potentially unsafe compounds based on the changes in mRNA expression patterns they induce. Among other purposes toxicogenomics should help in drug candidate selection in very early phases of the development process. Due to the limited amount of compound available in these early phases of development such investigations would require *in vitro* experimentation methods using isolated tissues, cells or cell lines. We therefore characterized several hepatic *in vitro* systems based on mRNA expression profiles, comparing them to gene expression in liver tissue. The *in vitro* systems investigated comprise two rat liver cell lines (BRL3A and NRL clone 9), primary hepatocytes in conventional monolayer or in sandwich culture, and liver slices.

The results demonstrate that pronounced gene expression changes take place over time in all the investigated primary *in vitro* systems. Expression patterns change most rapidly soon after cell isolation and culture initiation and stabilize with time in culture. Many genes were identified which exhibit strong changes in expression levels in all or at least one of the *in vitro* systems relative to whole liver. These gene expression changes inherent to the respective experimental system might mask or counteract changes induced by compound treatment. The findings are discussed with respect to the usefulness of hepatic *in vitro* systems for microarray-based toxicological testing of compounds.

S2-C8

USE OF HUMAN HEPATOCYTES TO MEASURE GLUCOCORTICOID RECEPTOR MEDIATED RESPONSES FOR CHARACTERISATION OF NOVEL ANTI-INFLAMMATORY COMPOUNDS.

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Synthetic glucocorticoids are powerful anti-inflammatory agents, but their use in chronic conditions is limited by side effects such as osteoporosis and diabetogenesis. The latter is largely driven by the stimulation of gluconeogenesis in the liver, where glucocorticoids upregulate a variety of metabolic enzymes. Of these, the regulation of tyrosine aminotransferase (TAT) is best understood, and this is widely used as a marker of glucocorticoid function in the liver. Glucocorticoids have several distinct molecular modes of action, and we have identified compounds which distinguish between these, retaining the ability to transrepress proinflammatory gene expression, but lacking the capacity to directly activate response genes. These molecules demonstrate efficacy in rat models of inflammation, but fail to induce TAT expression in either a rat hepatotoma cell line or rat liver in vivo. In order to characterise molecules in human systems, we have developed an assay for TAT expression using primary human hepatocytes. Treatment of hepatocytes with dexamethasone but not the antagonist RU486 results in significant increases in TAT expression. This system can be used to explore the activity of novel compounds.

CO-CULTURE OF ACTIVATED PRIMARY RAT STELLATE CELLS WITH RAT OR HUMAN HEPATOCYTES ON POLY-(DL-LACTIC ACID) TO RAPIDLY PRODUCE FUNCTIONAL CELL SPHEROIDS

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Introduction

Achieving sustained viability and differentiation of hepatocytes *in vitro* is a major challenge. *In vivo*, non-parenchymal cell types work synergistically with hepatocytes in a 3D architecture to constitute a functional system. In particular, the hepatic stellate cell has been identified as an important *in vivo* paracrine regulator of liver function and regeneration. This regulatory complexity is lost in standard culture. Consequently, we have developed a hepatic stellate cell – hepatocyte co-culture system in an attempt to reproduce some of these interactions *in vitro* and to create 3D tissue with *in vivo*-like morphological and functional attributes.

The Co-culture Technique

Activated primary hepatic stellate cells and freshly isolated hepatocytes are co-cultured on a low adhesion poly-(DL-lactic acid) surface on which they rapidly form well defined spheroids. Fluorescent marking of stellates (Cell Tracker^{TM}) and time lapse photography have allowed us to visualise the dynamic interaction of the cells during this process. The spheroids are dense structures relative to mono-culture spheroids (H&E staining), are approximately 100μm in diameter, and are viable after five days (Live-Dead™ staining visualised with confocal microscopy). The co-culture spheroids have an organised extracellular matrix support consisting of collagen (PSR stain) and reticulin (Silver stain) fibres throughout, and a fibronectin capsule (immuno-localisation). Organised ultrastructure such as bile canaliculi, tight junctions and desmosomes, are visualised with TEM (D). All such ECM and ultra-structure features are absent in mono-culture.

The co-culture system maintains higher levels of albumin production (ELISA) and cytochrome P450 1A1 activity (EROD assay) over a 35 day period compared with hepatocyte only spheroids. Testosterone metabolism (HPLC) is increased during the first 10 days of culture relative to both monolayer and spheroid mono-culture controls, demonstrating specific maintenance of P450 3A and 2B function. Testosterone metabolism is also more inducible in co-culture after 7 days. Furthermore, expression of P450 3A4 mRNA is improved throughout the first week of co-culture (Taqman). Preliminary work co-culturing human hepatocytes with rat stellate cells has demonstrated similar spheroid formation and improved testosterone metabolism.

S2-O6

AN IN VITRO SYSTEM FOR PREDICTING TOXICITY BY GENE EXPRESSION PROFILING

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Toxicogenomics can be used as a tool to analyze toxicology at the molecular level without preconceived notion on the mechanisms involved in the mode of action. Also, in comparison to conventional parameters for toxicity, the exact similar endpoint can be assessed both in vitro and in vivo. Comparison of toxin-specific expression profiles between these two situations makes it possible to determine the relevance of in vitro models in terms of their predictive value of molecular alterations associated with toxicity as they occur in vivo. In this study, CYP enzyme activities were compared between freshly isolated hepatocytes and hepatocytes cultured in sandwich configuration maintained for 72 hours in a standard and modified (to stabilize CYP enzyme activity) in vitro system by the testosterone hydroxylation assay. The modified in vitro system showed enzyme activities comparable to freshly isolated hepatocytes. After 3 days of culture, hepatocytes were exposed for 24 hours to coumarin, a compound from which hepatotoxicity is dependent on CYP conversion. Coumarin toxicity was found more severe in the modified in vitro model, determined by MTT reduction and LDH leakage assays. Toxicogenomics data for coumarin obtained from both in vitro models were compared to data obtained from an in vivo coumarin study. Principal component analysis (PCA) clearly separated the dose groups of the modified in vitro system and the in vivo study, but was unable to discriminate between dose groups of the standard in vitro system. In conclusion, the modified in vitro system can be a more suitable system for toxicogenomics studies predicting toxicity.

S2-O7

PGE1-DEPENDENT NITRIC OXIDE REDUCES D-GALACTOSAMINE-INDUCED CELL DEATH THROUGH ATTENUATION OF NF-KB ACTIVATION AND INOS EXPRESSION: IN VIVO AND IN VITRO STUDIES

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Introduction: Prostaglandin E1 (PGE1) has been shown to reduce experimental liver injury. Nitric oxide (NO) protects or exacerbates hepatocyte damage induced by different agents. Our study was focused on the role of NO during the cytoprotection by PGE1 against apoptosis and necrosis induced by D-GalN in culture rat hepatocytes. Material and Methods: PGE1 was pre-administered to D-GalN-treated male Wistar rats. The effect of NO or TNF-α; was evaluated by the administration of an inducible nitric oxide synthase (iNOS) inhibitior (methylisothiourea, MT) and anti-TNF- α; antibodies. The in vitro studies were carried out in cultured rat hepatocytes isolated by collagenase perfusion technique, and maintained in supplemented Williams'E culture medium. PGE1 was preadministered to D-GalN-treated hepatocytes. In this system, NO production was regulated by an iNOS inhibitor (N969;-nitro-L-arginine methyl ester, L-NAME) or a NO donor (S-nitroso-N-acetyl-penicillamine, SNAP). Different iNOS promoter regions fused to luciferase-reported gene including NF-kB sites were transfected to cultured hepatocytes. Apoptosis and necrosis were measured by DNA fragmentation and caspase-3 activation, an lactate dehydrogenase (LDH) release, respectively. NO end-products, nitrite+nitrate (NOx), were measured using the Griess reaction. The expression of iNOS was assessed by Western-blot and RT-PCR. NF-kB activation in nuclear extract was determined by EMSA. Results: PGE1-related reduction of apoptosis and necrosis by D-GalN was related to enhanced iNOS expression in rat liver, and this effect was prevented by MT. Anti-TNF- α; antibodies abolished the effect of PGE1 on iNOS expression and cytoprotection in D-GaN-treated rats. In vitro studies showed that PGE1 enhanced NF-kB activation, iNOS expression and NO production in control hepatocytes. But, PGE1 pre-administration reduced NF-kB activation, iNOS expression, NO production and apoptosis induced by D-GalN. L-NAME or low SNAP (0.0012-0.006 μM) reduced NO production and apoptosis by D-GalN. PGE1 enhanced and low SNAP concentration reduced the iNOS promoter activity including fully functionally NF-kB sites in transfected hepatocytes. Conclusions: 1) PGE1 reduced D-GalN-induced cell death in liver and in cultured rat hepatocytes. 2) The cytoprotective effect of PGE1 against D-GalN-induced apoptosis is related to the ability of the prostanoid to rapidly enhance NF-kB activation, iNOS expression and NO production in hepatocytes. 3) PGE1-derived NO was able to reduce the further increase of NF-kB activation, iNOS expression, NO production and apoptosis induced by D-GalN in hepatocytes.

MECHANISMS OF CANNABIDIOL INDUCTION OF HUMAN P450S: UTILISATION OF HEPG2-BASED REPORTER ASSAY AND PRIMARY HUMAN HEPATOCYTES

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A whole plant extract of cannabis containing Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) as the principal components is of therapeutic interest, particularly in the management of symptoms of multiple sclerosis. Recent work in our laboratory showed that repetitive administration of CBD (150 mg kg^{-1}) significantly increased rat hepatic CYP2B1 (140-fold) and moderately increased CYP3A23 (3.4-fold) mRNA expression. A significant increase in CYP2B1/2 protein (26-fold) and CYP2B1/2-dependent activity was also observed. CYP3A23 protein (2-fold) was weakly enhanced with no discernible increase in CYP3A-dependent activity when compared with control rats (corn oil). The induction of CYP2B1 and CYP3A23 by CBD appears to be regulated at the transcriptional level. This implicates the receptors CAR and PXR in the regulation of CYP induction by this plant cannabinoid. In addition, these results highlight the potential for CBD to induce the human orthologues, CYP2B6 and CYP3A4.

Primary human hepatocytes supplied by the Human Tissue Bank, De Montfort University, Leicester, UK and cultured on type I collagen in William's E medium supplemented with dexamethasone (100 nM) were exposed to CBD and THC covering a range of concentrations (0.5 to 250 μM) for 48 hours. Preliminary data indicate that human hepatocytes are less susceptible to the cytotoxic effects of CBD and THC when compared with rat hepatocytes. Following 72hours exposure to CBD (5 μM and 20 μM) CYP3A4 protein was modestly increased and CYP2B1/2 protein was weakly increased. In contrast THC produced no discernible change in the protein of either P450 under similar exposure conditions. To determine whether CBD could activate the human PXR, a cell-based reporter assay was employed. HepG2 cells transfected with the PXR response element (ER_6), human PXR and the luciferase reporter gene construct were treated with CBD (0.5 to 200 μM), rifampicin (10 μM) and phenobarbitone (1mM). The luciferase assay indicated that CBD is cytotoxic at concentrations exceeding 10 μM, which correlated with the results from the MTT assay. The ability of CBD to activate human PXR was variable between assays (n = 3), with an average 2-fold increase above control treatment (DMSO) at 10 μM. The activation profile for CBD was similar to rifampicin (10 μM) under the same assay conditions. Taken together these *in vitro* data suggest that CBD is capable of inducing human CYP3A4 and CYP2B6. As yet it is unclear as to whether activation of PXR by CBD plays a major role in this induction process.

S2-C9

DOWN-REGULATION OF CONNEXINS AND IMPLICATIONS FOR TOXICITY AND CARCINOGENESIS

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Connexin-mediated gap junctions play an important role in the control of cell homeostasis, differentiation and proliferation. There is evidence that connexins provide a tumour suppressor activity and their expression is controlled by a number of transcription factors. Down-regulation of connexin function (assessed by inhibition of inter-cellular communication in hepatocytes *in vitro*) can occur following chemical exposure via several different mechanisms which can be demonstrated at the transcriptional and post-translational level. In the short-term this is considered to be an adaptive, protective response but sustained down-regulation can contribute to tumour promotion *in vivo*. There is a strong correlation between disruption of connexin expression (demonstrated through immunohistochemistry) and tumourogenicity of non-genotoxic carcinogens particularly when seen in conjunction with a proliferative response. These effects may act as alerts (biomarkers) in the recognition of non-genotoxic carcinogens. In MH_1C_1 rat hepatoma cells *in vitro*, reduction of Cx32 using RNAi leads to elevation of proliferation. Furthermore, TNFα, in association with induction of apoptosis in these cells, also induces proliferation and loss of Cx32 plaque formation. The maintenance of Cx32 function in cells appears to be important in the control of proliferation and differentiation and this has important implications regarding maintenance of hepatocyte function *in vitro* as well as protection against carcinogenesis *in vivo*.

Reference:
Chipman JK Mally A and Edwards GO 2003 Disruption of gap junctions in toxicity and carcinogenicity, Toxicological Sciences, 71, 146-153.

S2-C10

USE OF CULTURED HEPATOCYTES TO SUPPORT SAFETY ASSESSMENT OF NEW PHARMACEUTICALS

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Toxicity to the liver is a major concern within the pharmaceutical industry because it may result in drug attrition during development and post-launch, complicate and delay project progression and drug registration. In addition, liver toxicity is being increasingly recognised as a significant cause of adverse drug reactions in man. This reflects an unmet need for new approaches that can be used in drug discovery, to guide drug design, support selection of efficacious and non-hepatotoxic drugs and to facilitate investigations of mechanisms of toxicity. We have been exploring the value of isolated hepatocytes in primary culture. Initially we explored the influence of cell culture conditions on viability and morphological appearance of hepatocytes isolated from Han Wistar rats by collagenase perfusion. Cells cultured as monolayers in collagen-coated plates exhibited flattened morphology, somewhat variable basal ATP levels and LDH leakage and a progressive decrease in viability in culture. Cells cultured in sandwich configuration rapidly established cell-cell contacts, acquired polarized morphology over several days with clear functional "canalicular pockets" and maintained high viability for at least 5 days in culture. Toxicity studies were undertaken using a selection of 18 hepatotoxic and 5 non-hepatotoxic compounds, using cell morphology, ATP content and LDH release as toxicity indices. The most striking feature of the results is that IC50 values for the 5 non-hepatotoxins exceeded 1.7mM, whereas IC50 values below 1.7mM were observed for 9 of the 18 hepatotoxins (50%). Similar IC50 values were obtained using the two culture systems, although the reproducibility of data obtained with sandwich cultures was markedly higher. These data suggest that in vitro toxicity studies with cultured hepatocytes may aid in selection of compounds having reduced hepatotoxic potential. For mechanistic investigations, a key first requirement is to establish that data obtained with cultured hepatocytes is relevant to the toxicity observed in vitro. Once this has been achieved, a broad range of mechanistic approaches can be applied. These include profiling metabolic activity alongside toxicity, exploring the influence on toxicity of modulating metabolism, assessment of cellular thiol status and quantification of irreversible (i.e. covalent) binding to protein, and investigation of transporter activity. Examples of practical use of these various approaches will be described.

S2-A6

HUMAN LIVER SPHEROID CULTURE: MORPHOLOGY AND FUNCTIONALITY

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Liver is an important target in biomedical studies and species differences are a major concern when data from animal experiments are extrapolated to humans. It has been demonstrated in rats that hepatocytes cultured as spheroids maintain superior functionality (1-2). Liver spheroid culture is also an effective way to make good use of scarce tissue, e.g. human. If human hepatocytes can be grown into spheroids and maintain functionality, this model would have the potential to offer a relevant paradigm and reduce animal use in research. This report investigates human liver spheroid culture by a gyrotatory method and evaluates morphological formation and functionality. Fresh hepatocytes were provided by the UK Human Tissue Bank. Spheroids formed gradually under gyrotation (83-77 rpm) and took 5-6 days to reach a size range of 160 ± 30 μm. After that, the size of spheroids tended to stabilise. Human liver spheroids maintained stable glucose secretion function and P450 1A metabolic activity (indicated by confocal microscopy after incubation with ethoxyresorufin). Urea secretion was initially high and thereafter decreased and was maintained at a relatively stable level 4 days after cell plating. Pyruvate uptake and γ-glutamyltranferase activity were maintained at relatively stable levels during a period of 7 days observation. It is concluded that human hepatocytes can form spheroids under gyrotation and the resultant spheroids maintain liver specific functionality.

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LONG-TERM 3D-CULTURE OF LIVER EXPLANTS IN RELATIVE MICROGRAVITY: NEW PERSPECTIVES AND POSSIBLE APPLICATIONS

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General Pathology & Immunology Unit, School of Medicine, University of Brescia, viale Europa 11 25123 Brescia, Italy Abstract=The achievement of reliable in vitro models of liver tissue alternative to the use of intact organisms is becoming a priority research objective in the actual scientific and industrial context. Responsible for the majority of liver-associated functions, hepatocytes represent about the 60% of the whole population of liver cells. When used in primary culture (i.e. monolayers of isolated cells) this complex cell type rapidly loses its highly differentiated phenotype and shows a limited survival rate. It is well known how cyto-architecture and heterotypic cell interactions greatly influence the differentiation state and the metabolic behaviour of cell cultures. To overcome the limits of traditional cell monolayers, several liver-derived in vitro systems, such as 2D-co-cultures and 3D-models (e.g. cell aggregates, spheroids or tissue slices), were developed; nevertheless, none of them has actually reached optimal characteristics for allowing the long-term preservation of the original tissue-like organisation and differentiated hepatic cell phenotype. Since it has been recently proved that the conservation of original cell-cell and cell-extracellular matrix interactions is of pivotal importance for the maintenance of cell integrity and functions, we developed a 3D liver-derived culture system based on the use of the Rotating Wall Vessel device (RWV, Syntecon) adapted to the direct culture of liver explants. Operating at a very low shear regimen, this innovative technology allows, in fact, the establishment of a particular microenvironment where hydrodynamic forces are strictly controlled (relative microgravity), thus providing a powerful tool for reproducing specific conditions for 3D-tissue morphogenesis. Once established, our model was then investigated for its suitability in the long-term maintenance of viable and physiologically differentiated hepatocytes. The preservation of the typical liver architecture, as well as the sustained expression of liver-specific structural and functional markers that, normally, are quickly lost in the in vitro conditions (e.g. liver-specific hormonal receptors and junctional proteins), confirmed the validity of our model. Moreover, when tested for its response to toxic conditions, the model demonstrated also a specific and high sensitivity. In conclusion, all these features make our 3D model a powerful tool for the in vitro study of liver-specific physio-pathological processes, as well as for the mechanistic investigation of toxicants and pharmaco-active compounds.

S2-A8

INFLUENCE OF DIFFERENT PARAMETERS ON CRYOPRESERVED HEPATOCYTES

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Cryopreservation of hepatocytes in suspension is suitable for the long-term storage with limited loss of viability. In contrast to cells in suspension, liquid nitrogen storage of monolayer and three-dimensional cultures is associated with an extensive decrease in cell viability due to altered biophysical properties of the cells in aggregates.
In a first set of experiments we examined the influence of four different cooling rates (5°C/min, 1°C/min and the same cooling rate with a plateau of 15 min at –7°C), two DMSO concentrations (5% and 7.5%) and different cryosubstrates (Fraunhofer-substrate, freezer bag and cryovials) on post-thaw viability and albumin synthesis of the human hepatoma cell line HepG2. For this purpose the viability of HepG2 cells in suspension, developed spheroids, cultivated in microstructures, designed for three-dimensional cell culture, was determined by fluorescein diacetate and propidium iodide or ethidium bromide staining. Post-thaw viability of monolayer-cultures was accomplished by LDH leakage quantification. In addition, albumin production was investigated over a period of three days after thawing.
Our preliminary data show, that a cooling rate of 1°C/min (with and without a hold time of 15 minutes) is suitable for the preservation of isolated cells and spheroids in Fraunhofer-substrates (25 µl) and HepG2 cells in suspension, monolayers and microstructures in freezer bags (5 ml). The freezing protocol with a 15 min plateau phase yielded a higher viability for HepG2 cells than the protocol with a constant cooling rate. The obtained viability of cryopreserved cells in suspension with both freezing protocols averaged to 70 ± 15% (n = 4) to 80 ± 11% (n = 4) depending on the cryosubstrate used. In contrast, post-thaw viability of cells cultured in spheroids, microstructures and monolayers was considerably lower and seemed to depend on both the cooling rate and DMSO concentration. Viability of monolayers frozen with both freezing protocols mentioned above and 5% (v/v) DMSO was about 34 ± 13% (n = 8) and with 7.5% (v/v) DMSO approximately 47 ± 19% (n = 8). Spheroids and cells cultured in microstructures frozen with 1°C/min and a 15 min hold time at –7°C upon thawing showed the highest viability(43 ± 5% to 58 ± 6%, n = 4). Analysis of daily LDH leakage from cryopreserved cells in suspension and monolayers displayed a basal loss of LDH of up to 45% within 24h. Cells frozen as monolayers showed an increase in LDH release 24h after thawing compared to cells in suspension probably due to a loss of membrane integrity. However, albumin production for monolayers was markedly higher than the production by cryopreserved cell suspension. This work is supported by a BMBF-Grant No 16SV1366/0 given to H.Z.

REACTION PHENOTYPING USING BOTH DRUG DEPLETION AND METABOLITE FORMATION

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An in vitro approach was setup to elucidate the enzymes responsible for the metabolism of promising candidate drugs using human liver microsomes and recombinant human cytochrome P450 (CYP) and flavin-containing monooxygenase (FMO) isoenzymes. The depletion of the parent drug and the formation of expected metabolites were followed every 10 minutes starting at five different concentrations for 50 minutes using LC-MS/MS. The metabolic intrinsic clearance observed by means of drug depletion for every recombinant enzyme was scaled up towards the whole human enzyme content to predict the contribution of the different enzymes, over a broad concentration range. The formation of metabolites, analyzed simultaneously with parent drug depletion, was used to confirm parent drug depletion results.
Information obtained can be used to anticipate on drug interaction studies and can help to interpret non-linear kinetics and polymorphism in the clinic.

S2-A10

PLASMA-BASED SURFACE MODIFICATION FOR TISSUE ENGINEERING PURPOSES: THE EFFECT ON HEPATOCYTE ATTACHMENT AND FUNCTIONALITY.

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Plasma surface modification has been used for many years in the fabrication of tissue culture plastics [1, 2]. Using the related method of plasma deposition, glass can be surface modified to improve cell attachment for tissue engineering purposes. Glass was either coated with collagen gel (0.02%), plasma treated with allylamine (100nm thickness) or plasma treated with allylamine and coated with collagen gel (0.02%). All samples were compared to untreated glass as a control. X-Ray photoelectron spectroscopy (XPS) showed an increase in the concentration of nitrogen containing amine/imine and amide groups on the surface of the glass. Phase contrast microscopy demonstrated cellular attachment was improved after allylamine plasma treatment, collagen gel coating or allylamine plus collagen gel when compared to control glass after 24 hours and 48 hours. The hepatocyte ethoxyresorufin-O-deethylase (EROD) activity after 24 hours was approximately two fold higher on allylamine treated glass compared to the control. The results indicate allylamine plasma surface modification improved attachment and function of hepatocytes compared to glass

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OPTIMIZATION OF THE TECHNIQUE OF HYPOTHERMIC STORAGE OF EQUINE ISOLATED HEPATOCYTES

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Recently, a new method of hepatocytes isolation from slaughtered horse has been introduced (1). Major advantages of the new method are obtaining the organs from routinely slaughtered animals at the slaughterhouse and a high cell yield from a single preparation, sufficient for a large scale in vitro assay. Unfortunately, the use of equine hepatocytes may be limited because of the long distance between slaughterhouses and laboratories. This is a particularly significant problem, because daily transportations of equine livers from slaughterhouse to laboratory is labor and time consuming and thus increases the costs of cell preparations. The ability to store isolated hepatocytes would reduce the necessity of every day the organs transportation to the laboratory and thus would greatly facilitate biomedical research. Moreover, storage of cells, either in suspension or in primary culture, is highly desirable for in vitro investigations on equine hepatocytes because related experiments can be performed without dependence on the time and logistics of the cell isolation process. Therefore, there is a need for elaboration of optimal methods of hypothermic storage of isolated hepatocytes. We have investigated the effects of preservation time, cell density, and type of medium used for hypothermic storage of hepatocyte suspensions on equine isolated hepatocytes viability, metabolic activity, and morphological appearance. Materials and methods Hepatocytes were isolated from slaughtered horses (4 to 13 years of age, both sexes, various breeds) as described previously (1). Hepatocyte suspensions were judged suitable for preservation if, as determined microscopically, over 85% of cells were viable in trypan blue exclusion (TB) and 5,6-carboxyfluorescein diacetate (CFDA) tests. Immediately after viability determination, cells were washed twice (80 x g for 2min) in the following storage solutions: HBSS, WE or UW. Finally, cells were resuspended at cell densities of 0.5, 2.5, 5 and 12.5 x 106 viable cells/ml inappropriate storage solution (Hanks Balanced Salt Solution HBSS, Williams' Medium E -WE and University of Wisconsin Solution - UW) and stored at 4°C for 0, 24, 48 and 72 hours. After storage cell morphology was investigated under light microscopy and next cell suspensions were washed (80 x g for 2 min) in culture medium (WE supplemented with 5% Fetal Bovine Serum) prior to culturing. Cell viability was assessed by TB and CFDA tests. Functional integrity of isolated hepatocytes was determined with MTT reduction test and microscopic evaluation of cell attachment rate. For determination of cell attachment rate and MTT reduction hepatocytes were seeded on 96-well plates (5 x 104 viable cells per well) for 10 h at 37şC in 5% CO2/95% air. Results Viability of hepatocytes assessed with TB was 32.5,

49.2, 75.2% after 24 h storage, 17.3, 22.8, 68.2% after 48 h and 8.1, 12.3, 50.4% after 72 h storage in HBSS, WE and UW, respectively. Viability determined with CFDA was slightly lower from this stated with TB. After storage in HBSS and WE, cells attached poorly and exhibited very low MTT reduction, with zero values after 48 h storage. In contrast, hypothermic storage of equine isolated hepatocytes in UW resulted in well-preserved cells for up to 48 h storage. After 24 h storage of isolated hepatocytes in UW cell viability decreased slightly to about 70% and cell attachment rate and MTT reduction were only 8 and 13% lower than observed in freshly isolated cells, respectively. There was also observed a significant effect of the cell density on viability and metabolic activity of hepatocytes. Higher cell densities did improve cell survival during hypothermic storage and the effect was more visible during cell storage in UW. After 24 h, viability of hepatocytes stored at densities of 12.5 x 106 and 30 x 106 cells/ml was not significantly affected (determined by TB exclusion and CFDA metabolism test), whereas at 2.5 x 106 cells/ml considerably declined to 59.2 ? 4.5%. There was also correlation between storage time, medium composition and changes in histological appearance of isolated hepatocytes. Hepatocytes, after 24 h of storage in UW solution at density of 12.5 x 106 cells/ml, showed typical morphology with low translucent blebbing of plasma membrane. In contrast, cold storage of hepatocytes in HBSS and WE for 24 h resulted in a drastic increase of the number of bleb-bearing cells. Also, reduction of hepatocyte viability during preservation in UW solution for 48 and 72 h was correlated with an increase in the number of bleb-bearing cells (to 35.9 ? 9.1 and 59.4 ? 16.1% after storage for 48 and 72 h, respectively). The results indicate that equine isolated hepatocytes can be stored in UW at a cell density of 12.5 x 106 viable hepatocytes/ml for 24 h without significant decrease of hepatocytes viability and metabolic activity.

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INTRODUCING THE MEASUREMENTS FOR BIOTECHNOLOGY PROGRAMME 2004-07

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The Measurements for Biotechnology programme (http://www.mfbprog.org.uk) is a DTI sponsored initiative that has three key objectives:

- to improve the accuracy and reliability of biomeasurements important for industry;
- to strengthen the measurement science underpinning the regulatory regime for biotechnology;
- to ensure that the UK biomeasurement system is co-ordinated and developed in harmony with those of other countries.

From 2004, there are four project themes, namely cell-based technology, gene measurement, product characterisation and protein measurement. Technical projects under each of these themes are collaborative undertakings involving academics, SMEs and large companies and will foster knowledge transfer between these organisations and the regulators.

Under the cell-based technology theme, LGC is collaborating with Abcellute and the UKHTB to develop and establish biomarkers that signify "fitness for purpose" in hepatocytes. These biomarkers may then be used as a basis on which to accept or reject data from a particular batch of hepatocytes, and may be functional, biochemical, genetic, morphological or otherwise in nature. The aim will be to devise a standard set of biomarkers with protocols and measurement guidance to aid in the use of human hepatocytes.

It is acknowledged that at the present time most institutions and companies operate their own internal quality controls, or batch testing, on hepatocytes from internal and external sources. The project aims to draw on this expertise while undertaking basic research, intially using rat hepatocytes and subsequently with human hepatocytes, to distil this knowledge into workable guidelines that may be accepted by the regulator and be of direct benefit to UK industry.

At this stage, we are looking to develop a field consensus on the most appropriate biomarkers for study. Previous work under the MfB 2002-2004 programme included the analysis of 4 major metabolites of testosterone (6β, 7α, 16α and 16β-OH) and corresponding expression of the four P450 enzymes involved in its metabolism (3A2, 2A1, 2C11, 2B1) by RT-PCR. Data from these studies indicated that expression analysis was difficult to correlate with metabolite production, but that both declined with time in culture and useful information about the state of the cells could be generated.

All parties are invited to submit opinions, and a questionnaire form will be available for completion.

LIVER GRAFTS PRESEVED IN CELSIOR SOLUTION AS SOURCE OF HEPATOCYTES FOR DRUG METABOLISM STUDIES: COMPARISON WITH SURGICAL LIVER BIOPSIES.

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The expansion of the liver transplantation programmes has contributed to the increasing availability of human liver tissue for research purposes, as donor livers that cannot be implanted for different reasons can be used for hepatocyte isolation. In contrast to tissue samples from other sources, liver from organ donors are perfused in situ with a cold preservation solution to avoid warm ischemia and the tissue is usually transported and maintained under these conditions for several hours until hepatocyte isolation. Cold ischemia is a risk factor for organ function and it could be also considered as a possible factor involved in the efficiency of the isolation procedure and the metabolic competence of cultured cells. Suitability of human liver grafts preserved in Celsior solution (CS) for preparing metabolically competent hepatocyte cultures has been examined. To this end, basal and induced activity and mRNA levels of major hepatic cytochrome P450 (P450) enzymes have been measured. By 24 h in culture, measurable levels of the ten P450 mRNAs studied were found in all hepatocyte preparations examined, with CYP2E1, CYP2C9 and CYP3A4 mRNAs being the most abundant. Compared to hepatocytes obtained from surgical liver resections (SLR), lower content of each P450 mRNA was found in hepatocytes from the CS group, however, the relative distribution of individual P450 mRNAs was similar. Similar results were observed after measuring P450 activities. CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2E1, and CYP3A4 activities in hepatocytes from CS-flushed grafts were lower, but comparable, than those of cultures prepared from SLR. No differences in the metabolite profile of testosterone were found. Treatment of hepatocytes from CS-preserved grafts with model P450 inducers shows that 2 mM methylcholanthrene only increased CYP1A1 and CYP1A2 mRNAs (>100-fold over control); 1 mM phenobarbital markedly increased CYP2A6, CYP2B6 and CYP3A4 mRNA content (>7-fold); and 50 mM rifampicin highly increased CYP3A4 mRNA levels (>10-fold), whereas minor effects (<3-fold) were observed in CYP2A6, CYP2B6, and CYP2C9 mRNAs. This induction pattern of P450s was similar, in terms of magnitude, reproducibility and specificity to that shown in primary hepatocytes from surgical biopsies. Overall, our results indicate that cold-preserved in CS liver grafts constitute a valuable source of human hepatocytes for drug metabolism studies.

TESTOSTERONE HYDROXYLATION BY HUMAN HEPATOCYTES WITH AND WITHOUT CRYOPRESERVATION

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Human hepatocytes have been isolated from surgical resections, cultured in a sandwich gel configuration and tested for their ability to metabolize testosterone by analysis of testosterone hydroxy derivatives in the culture supernatant. For determination of testosterone hydroxylase activity, the cultures were incubated with cell culture medium containing 250 µM testosterone for a period of 24 hours. For induction, rifampicin (50 µM) was present for a period of 24 or 48 hours. Generation of 6β-hydroxytestosterone (6β-OHT), 2α-OHT, 2β-OHT, 6α-OHT, 7α-OHT and 16α-OHT was quantitated using an sensitive automated "on-line" pre-column sample enrichment RP-HPLC assay with corticosterone as the internal standard. All six testosterone metabolites could be sufficiently separated from each other to achieve an unequivocal assignment to the individual structures. A total of 15 donors was analyzed for 6β-testosterone hydroxylase activity in fresh cultures. Activity during long-term culture (up to 15 days) is reported together with the induction factors. Individual metabolism and induction patterns of 6β-OHT, 2α-OHT, 2β-OHT, 6α-OHT, 7α-OHT and 16α-OHT are also presented for 3 of the donors. In addition, testosterone hydroxylation was analyzed after cryopreservation following a new cryopreservation protocol. Most of the cultures could be analyzed only at day 1 after thawing, but two cultures could also be analyzed at day 3. 6β-OHT, 2β-OHT, 6α-OHT, 7α-OHT and 16α-OHT activities could be determined. We report the actual testosterone hydroxylation activities together with the induction factors and the cryopreservation recovery rates.

UTILITY OF LONG-TERM CULTURED HUMAN HEPATOCYTES AS AN *IN VITRO* MODEL FOR CYTOCHROME P450 INDUCTION

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Many xenobiotics can induce cytochrome P450 enzyme expression, and induction of these enzymes may have considerable implications for drug therapy. Therefore, understanding the induction potential of a new chemical entity at an early stage in discovery is crucial. However, availability of viable tissue often limits evaluation of induction potential in human hepatocytes. A solution is to increase the time period during which the tissue remains viable. In this study we have investigated the induction of several CYP450 isozymes in long term cultured hepatocytes compared with fresh hepatocytes. CYP3A4, 1A1, 1A2, 2B6 and 2C9 are shown to be inducible in long term cultured hepatocytes. The inducibility of the different isozymes is comparable to that observed in fresh hepatocytes. The fold induction varied between donors, and between short and long term cultured hepatocytes from the same donor. This variability can be controlled by normalising data from each hepatocyte preparation to a positive control. Long term cultured human hepatocytes can be used to investigate CYP450 induction potential in a 96-well plate format.

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