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The Effects of Immunosuppressive Agents on the Function of Human Hepatocytes In Vitro

Trinidad Serrano,[1] Ragai R. Mitry, Claire Terry, Sharon C. Lehec, Anil Dhawan, and Robin D. Hughes

King's College London School of Medicine at King's College Hospital, Institute of Liver Studies, London, UK

Calcineurin inhibitors (tacrolimus) and steroids continue to be an important component of hepatocyte transplantation protocols, despite reports of hepatotoxicity and inhibitory effects of steroids on cell proliferation. The aim of the study was to investigate whether isolated human hepatocytes were more vulnerable to the toxicity of these agents and also to investigate their effects on hepatocyte VEGF secretion, a vascular permeability factor suggested to be involved in the cell engraftment process. Human hepatocytes were isolated from donor livers/segments rejected or unused for orthotopic liver transplantation using a collagenase perfusion technique. Hepatocytes were plated for cell function tests and to determine VEGF production. Tacrolimus (0–50 ng/ml) and methylprednisolone (0–500 ng/ml) were added to the culture media and cells incubated for 24 h. Cell metabolic activity was assessed using the MTT assay, cell number using the SRB assay, and cell attachment from hepatocyte total protein content and protein synthesis using [^{14}C]leucine incorporation. VEGF in culture supernatants was measured by ELISA. Tacrolimus and methylprednisolone had no statistically significant inhibitory effects on metabolic activity or protein synthesis compared to controls at all concentrations of the agents tested when added after plating. There were also no significant effects on cell attachment when tacrolimus or methylprednisolone was added at the time of cell plating. There were no differences in the responses obtained when either fresh or cryopreserved hepatocytes were used. The amount of VEGF secreted by untreated hepatocytes was highly variable (0–1400 pg/10^6 cells/24 h). VEGF levels in the culture supernatant from hepatocytes isolated from ≤20-year-old donors (687 ± 59 pg/10^6 cells/24 h) was significantly greater than from older donors (61 ± 7 pg/10^6 cells/24 h; $p = 0.003$). Tacrolimus and methylprednisolone did not significantly affect VEGF secretion by hepatocytes. Tacrolimus and methylprednisolone did not have detrimental effects on the metabolic function of human hepatocytes, cell attachment, or VEGF secretion after cell isolation.

Key words: Hepatocytes; Tacrolimus; Methylprednisolone; Transplantation; Vascular endothelial growth factor

INTRODUCTION

Hepatocyte transplantation is being increasingly used as a technique for bridging patients to whole-organ transplantation, providing metabolic support during liver failure, and replacing whole-organ transplantation in certain metabolic liver diseases. Isolated hepatocytes obtained from unused donor livers are infused into the portal vein or the spleen, in order to replace the deficient liver function(s). As with whole-organ transplantation, immunosuppressive agents are required for allogenic hepatocyte transplantation. Although some experiments have demonstrated less of an immune response against isolated hepatocytes than in liver transplantation, similar immunosuppressive regimens are used for hepatocyte transplantation (1,3,5,8,13,16,29).

The immunosuppressive agents can have cytotoxic effects in the liver, which has been shown in rats (27) and also with rat hepatocytes (7), but there are little data with human hepatocytes. Tacrolimus (FK-506) has been described as both a cytotoxic and cytoprotective molecule and is associated with development of vascular injury as a result of endothelial cell toxicity. On the other hand, tacrolimus protects rat hepatocytes against hypoxic injury by preventing mitochondrial dysfunction (14) and also prevents Fas-induced apoptosis in human hepatocytes (10). Corticosteroids are also currently used for immunosuppression in hepatocyte transplantation.

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[1]Present address: Transplant Unit, Lozano Blesa University Hospital, Zaragoza, Spain.
Address correspondence to Dr. Robin Hughes, Institute of Liver Studies, King's College London School of Medicine, Bessemer Road, London SE5 9PJ, UK. Tel: +44 (0)20 3299 3137; Fax: +44 (0)20-3299-3760; E-mail: robin.hughes@kcl.ac.uk

Methylprednisolone has inhibitory effects on liver regeneration in rats after partial hepatectomy (15), which has implications for hepatocyte repopulation after hepatocyte transplantation, whereas cyclosporin A and tacrolimus may both increase hepatocyte proliferation (9).

On infusion into the portal circulation transplanted cells need to become lodged in the hepatic microvasculature, enter the liver sinusoids, and then integrate into the hepatic parenchyma. In this process hepatocytes will embolize the portal bed, producing microcirculatory perturbations, portal hypertension, and ischemia-reperfusion injury. These events to a certain extent help increase endothelial permeability and disruption required for transplanted cells to enter into the liver plate, but if too many cells are infused this could be deleterious (11). One of the endogenous factors that may be released and contribute to these events is vascular endothelial growth factor (VEGF). This mediator increases vascular permeability and communicates between hepatocytes and sinusoidal endothelial cells (12). Under hypoxic conditions VEGF receptors on endothelial cells are upregulated (6). Hepatic sinusoidal dilatation will increase the entry of transplanted cells into the liver plate, improving cell engraftment and decreasing microcirculatory perturbation (26). Recent work has demonstrated that VEGF increases survival of transplanted hepatocytes within the hepatic portal radicles (24). Modulation of VEGF production could therefore influence the cell engraftment process and repopulation of the recipient liver. Corticosteroids have been shown to have inhibitory effects on VEGF gene expression in human vascular smooth muscle cells in vitro (20). Similar effects on hepatocytes could be relevant to hepatocyte transplantation. The effect of tacrolimus on VEGF production by cells is not known.

The aim of the study was to investigate whether the immunosuppressive drugs (tacrolimus and methylprednisolone) have effects on metabolic activity, cell attachment, and VEGF secretion of human hepatocytes in vitro.

MATERIALS AND METHODS

Preparation of Isolated Human Hepatocytes

Human liver tissue was obtained from 13 donor livers (8 male, 5 female) rejected or unused for orthotopic liver transplantation at King's College Hospital, London. The mean donor age was 30.2 ± 21.4 (SD) years.

Cells were isolated using a collagenase perfusion technique according to Strom et al. (28) with some modifications (17). Briefly, tissues were perfused at 37°C with buffer solutions: Hanks's balanced salt solution (HBSS, Bio Whittaker UK, Berkshire, UK) containing 0.5 mM EGTA, then HBSS and finally Eagle's modified essential medium (EMEM) (Cambrex UK) containing collagenase P at 500 mg/L (Roche Diagnostics Ltd., East Sussex, UK). The digested tissue was minced in ice-cold EMEM containing 2% human serum albumin (HAS) and filtered through sterile gauze. Hepatocytes were purified by washing two times with ice-cold EMEM and 2% HAS and once with ice-cold M199/HEPES containing 4% HAS using low-speed centrifugation ($55 \times g$, 5 min at 4°C). Cell yield and viability were determined using a hemocytometer and the trypan blue exclusion technique. In some cases cells were immediately cryopreserved in UW solution containing 10% DMSO using a controlled-rate freezer (30).

Culture of Hepatocytes

Freshly isolated or thawed hepatocytes were plated in 96-well collagen-coated plates (50,000 cells/well) for the cell metabolic function assays and in six-well plates ($2–3 \times 10^6$ cells/well) for VEGF production. The standard culture conditions consisted of Williams' E medium (WEM) containing 10% fetal calf serum (FCS), penicillin (50 U/ml) and streptomycin (50 μg/ml), and L-glutamine (2 mM). Cultured hepatocytes were maintained at 37°C in 95% O_2 and 5% CO_2.

Immunosuppressive Agents

Tacrolimus and methylprednisolone were obtained from Sigma-Aldrich (Dorset, UK). Concentrations of the agents used were based on levels targeted in clinical practice: 10, 20, 30, 40, and 50 ng/ml in tacrolimus experiments and 100, 200, 300, 400, and 500 ng/ml in methylprednisolone experiments. Two sets of experiments were performed: 1) to study the effect of immunosuppressive agents on hepatocyte function, tacrolimus and methylprednisolone were added 24 h after plating to preattached cells; 2) to study the effect on cell attachment, agents were added at the same time as the hepatocytes were plated.

MTT Assay

The method of Oka et al. (21) was used with some modifications (18), which measures the metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-biphenyl tetrazolium bromide to an insoluble formazan by mitochondrial dehydrogenases only present in viable cells. Briefly, the culture medium was removed by aspiration and replaced with 100 μl of fresh medium. Then 20 μl of MTT solution was added per well. The plates were incubated at 37°C for 4 h. The medium was then removed by aspiration and 20 μl of trypsin was added to each well to detach the cells. Then 100 μl of isopropanolol/HCl was added per well and the plate was shaken for 30 min. The OD was immediately measured at 630 nm using a Dynex MRX Microplate Reader.

Sulphorhodamine B (SRB) Assay

This assay was carried out according to Mitry et al. (19) and was used as a colorimetric measure of cell number. After 24 h of culture, the hepatocytes were washed with ice-cold PBS and 200 µl of fresh culture medium was added to each well and 50 µl ice-cold 50% trichloroacetic acid solution was layered on top. After 1 h at 4°C to fix the cells, the wells were rinsed five times with tap water. The plate was then incubated at room temperature for 10 min with 100 µl 0.4% SRB solution and then rinsed five times with 1% acetic acid. When the wells were dry 200 µl of unbuffered Tris-base solution (20 mM, pH 10–10.5) was added to each well. The plate was placed on a gyratory shaker for 1 h and OD read at 550 nm.

Cell Attachment

The cell protein content was determined using the method of Lowry with modifications to facilitate the use of cultured cells in 96-well plates (30). For the total number of cells added, the plate was centrifuged at $100 \times g$ for 15 min and the medium removed carefully and protein concentration determined. For attached cells, the cultured hepatocytes were washed with ice-cold PBS to remove nonattached cells and protein concentration measured. The protein in attached cells was expressed as percentage of the total cell protein added.

[^{14}C]Leucine Incorporation

[^{14}C]Leucine (0.2 µCi per well; Amersham International, Buckinghamshire UK) in culture medium was added to each well of a 96-well plate containing treated or control hepatocytes. After 24 h the cells were harvested and transferred onto a glass fiber filter washing away excess [^{14}C]leucine, using a FilterMate™ Cell Harvester (Packard Instruments, Beaconsfield, Buckinghamshire, UK). The filter was left to dry thoroughly overnight before counting the [^{14}C]leucine present on the filter, using a Matrix™ 9600 Direct β-Counter (Packard Instruments).

VEGF Secretion

The culture supernatant was removed from the wells and stored at −80°C until the assays were performed. VEGF levels in the supernatants were measured using a quantitative sandwich ELISA detection kit, according to the manufacturer's instructions (Quantikine human VEGF, R&D Systems, Oxfordshire, UK). The results were expressed in pg VEGF per million of viable cells per 24 h of incubation.

Statistic Analysis

Results were analyzed by two-way ANOVA. Differences between the group means were considered significant at a value of $p < 0.05$. Results are presented as mean ± SE.

RESULTS

The viability of fresh hepatocytes was 72.2 ± 3.1% ($n = 9$) and 51.6 ± 1.9% ($n = 6$) in thawed cells. None of the hepatocyte function assays were significantly altered by the addition of up to 50 ng/ml tacrolimus and 500 ng/ml methylprednisolone (Table 1). Thus, MTT, SRB, and protein synthesis ([^{14}C]leucine incorporation) did not show any significant changes after incubation of the hepatocytes with different concentrations of agents for 24 h. Fresh and cryopreserved hepatocytes showed no difference in response to the two agents.

In the second set of experiments, cell attachment on plating was also not significantly altered by the presence of different concentrations of tacrolimus and methylprednisolone (Table 1). The attached cells, measured by cell protein, were similar with tacrolimus and methylprednisolone-treated hepatocytes as in untreated cells.

The amount of VEGF secreted by the different hepatocyte batches was highly variable (0–1400 pg/10^6 cells/24 h) and was found to be related to the age of the donor (Fig. 1). The amount of VEGF in the hepatocyte supernatants from donors of 20 years old or younger was 10-fold higher than from donors older than 20 years (687 ± 59 pg/10^6 cells/24 h vs. 61 ± 7 pg/10^6 cells/24 h; $p = 0.003$). There were no correlations of hepatocyte VEGF secretion with other factors such as sex of donor, viability of cells, attachment, or functional tests. Baseline VEGF secretion was not significantly different between fresh and thawed cells (196 ± 56 pg/10^6 cells/24 h vs. 188 ± 76 pg/10^6 cells/24 h).

The addition of tacrolimus at the range of concentrations tested did not affect VEGF levels in the hepatocyte supernatants after 24-h incubation (Fig. 2). There appeared to be a tendency for VEGF production to be decreased by increasing concentrations of methylprednisolone, but the differences were not statistically significant (Fig. 3).

DISCUSSION

The results of this study showed that tacrolimus and methylprednisolone at the concentrations tested did not have significant effects on hepatocyte metabolic activity or cell attachment efficiency in vitro. The latter is considered important in the context of hepatocyte transplantation, as cells need to attach to the hepatic endothelium as an initial part of the engraftment process. Both agents studied are commonly used in liver transplantation protocols and have also been used in the clinical studies with hepatocyte transplantation (1,3,5,8,13,16,29). This lack of effect was seen in both fresh and thawed hepatocytes, indicating that the cryopreservation and thawing

Table 1. Results of Metabolic Function Assays and Attachment of Isolated Human Hepatocytes Treated With Tacrolimus and Methylprednisolone

Tacrolimus	Control	10 ng/ml	20 ng/ml	30 ng/ml	40 ng/ml	50 ng/ml
MTT (OD units)	0.26 ± 0.05	0.24 ± 0.03	0.21 ± 0.03	0.23 ± 0.04	0.23 ± 0.03	0.23 ± 0.04
SRB (OD units)	2.33 ± 0.29	2.57 ± 0.23	2.56 ± 0.25	2.60 ± 0.22	2.39 ± 0.26	2.50 ± 0.23
^{14}C (counts/min)	50.1 ± 10.9	44.6 ± 9.8	44.2 ± 8.9	43.6 ± 9.6	41.3 ± 9.2	44.6 ± 12.9
Attachment (%)	57.7 ± 6.0	61.9 ± 6.1	60 ± 4.7	55.6 ± 6.5	58.1 ± 7.3	54.2 ± 5.2
Methylprednisolone	Control	100 ng/ml	200 ng/ml	300 ng/ml	400 ng/ml	500 ng/ml
MTT (OD units)	0.18 ± 0.04	0.17 ± 0.03	0.16 ± 0.03	0.17 ± 0.03	0.17 ± 0.03	0.16 ± 0.03
SRB (OD units)	1.93 ± 0.42	1.54 ± 0.36	1.88 ± 0.37	2.05 ± 0.38	2.08 ± 0.37	1.86 ± 0.41
^{14}C (counts/min)	28.5 ± 17	25.9 ± 12.5	29.5 ± 18.6	24.8 ± 19.2	27.5 ± 16.6	21.5 ± 11.8
Attachment (%)	56.9 ± 6.4	58.9 ± 6.4	64.3 ± 7.9	64.2 ± 7.9	61.2 ± 6.1	60.5 ± 6.1

Results are presented as mean ± SE of eight wells in at least five different experiments. ^{14}C = [^{14}C]leucine.

process does not increase susceptibility to possible effects of the immunosuppressive drugs. This is important as cryopreserved cells are the best source for hepatocyte transplantation for emergency use in hepatic failure and repeated treatment of patients with hepatocytes from the same donor.

Human hepatocytes isolated from unused donor livers preserved in UW solution secreted VEGF after the isolation process and on warm reoxygenation in culture. With rat hepatocytes it was found that warm reoxygenation after cold ischemia triggered VEGF mRNA expression (2). Some VEGF mRNA expression was observed after simple cold storage, whereas marked upregulation occurred after warm reoxygenation. In a rat model of hepatocyte transplantation VEGF was secreted by both native and isolated hepatocytes a few hours after the cells were transplanted (12). VEGF produces vascular dilatation and it is also known that hepatic sinusoidal dilatation by different drugs, such as nitroglycerine, increases the entry of transplanted cells into the liver sinusoids, improves cell engraftment, and decreases microcirculatory perturbations (26). This suggests that VEGF could play an important role in hepatocyte engraftment after cell transplantation. The present study has shown that tacrolimus did not affect VEGF secretion in isolated hepatocytes during 24 h of cell culture. Tacrolimus was recently shown to increase VEGF expression in the kidney glomeruli of transgenic hypertensive rats, but did

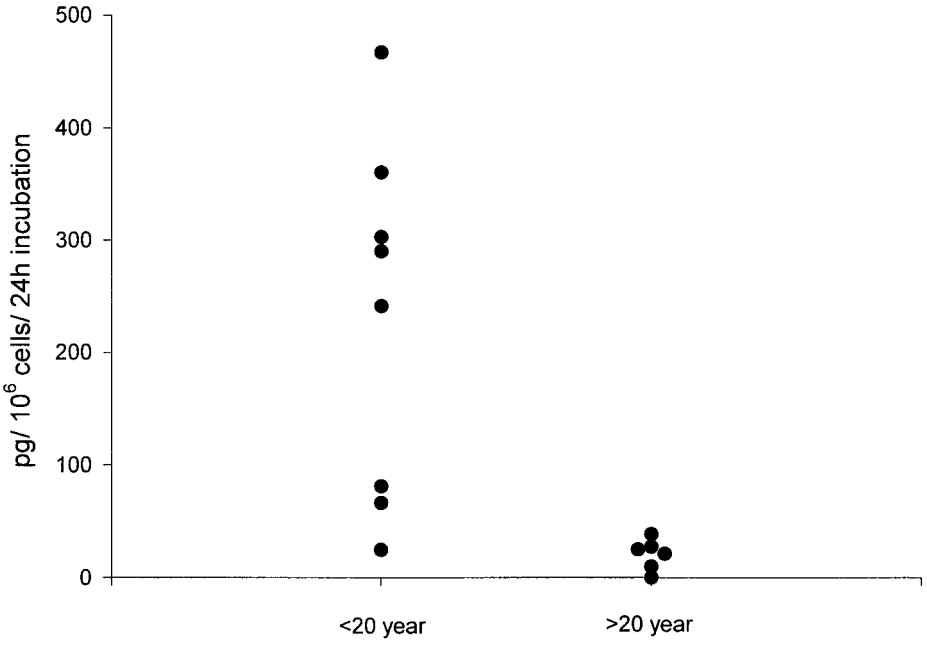

Figure 1. VEGF secretion by isolated human hepatocytes in relation to donor age.

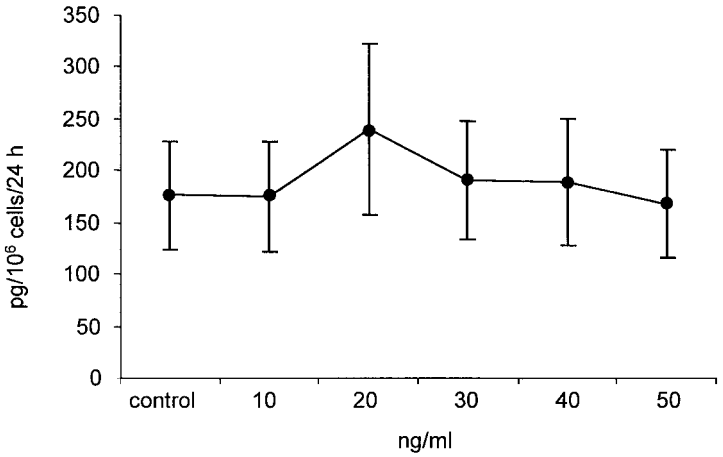

Figure 2. VEGF secretion by isolated human hepatocytes treated with tacrolimus.Results are presented as mean ± SE of eight wells in eight different experiments.

not have effects on healthy animals (22). Tacrolimus and cyclosporine have inhibitory effects on VEGF secretion by synovial fibroblasts from patients with rheumatoid arthritis (4), but the effect of these agents on synovial cells from healthy individuals was not studied. The inhibition could be related to the presence of cellular inflammation. Most liver donors do not have fibrosis or inflammatory disease, though there may be an inflammatory response to the transplanted hepatocytes in the liver. Treatment with corticosteroids decreased VEGF expression in hypertrophic chondrocytes in a rat model of chronic renal failure (23). In the present study methylprednisolone produced a slight decrease in VEGF levels, but this was not statistically significant. An inhibitory effect on VEGF secretion could produce detrimental effects on engraftment after liver cell transplantation. In islet transplantation steroid-free immunosuppression is used (25) and this may also be advantageous in hepatocyte transplantation.

The amount of VEGF secreted by hepatocytes was highly variable in the different batches of cultured cells. Other authors have reported that a high variability in VEGF secretion is related to specific genetic polymorphisms (32). In this study the variability appeared to be related to the age of the donor of the liver. Cells isolated from donors less than 20 years old had a 10-fold greater VEGF production when compared to older donors. Mitry et al. found a higher viability in hepatocytes isolated from younger donors (17) and such cells are also likely to have a greater regenerative potential (31). These findings taken together would suggest that isolated hepatocytes from young donors, perhaps not surprisingly,

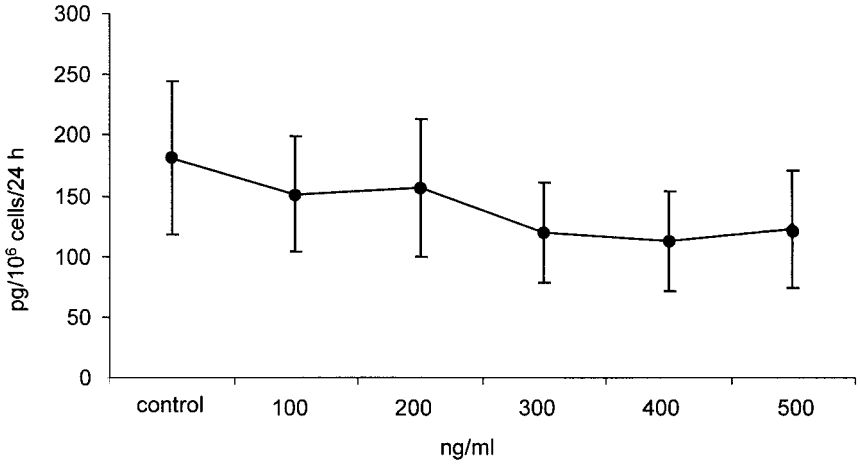

Figure 3. VEGF secretion by isolated human hepatocytes treated with methylprednisolone. Results are presented as mean ± SE of eight wells in eight different experiments.

might give better engraftment than those obtained from older donors. Further studies are necessary to investigate this hypothesis.

In summary, these experiments suggest that tacrolimus and methylprednisolone are not likely to have deleterious effects on hepatocytes immediately after transplantation, at a time when cells would be particularly vulnerable.

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