

HOMOCYSTEINE ACCUMULATION IN STIMULATED PROLIFERATING HUMAN PBMC AND DOWN-REGULATION BY ASPIRIN

Schröcksnadel K.¹, Frick B.¹, Wirleitner B.¹, Winkler C.^{1,2}, Fuchs D.^{1,2}

¹*Institute of Medical Chemistry and Biochemistry, University of Innsbruck*, ²*Ludwig Boltzmann Institute of AIDS Research, Innsbruck, Austria*

Moderate hyperhomocysteinemia is an established risk factor for atherosclerosis, thrombosis and also neurodegenerative and malignant disease. Elevated homocysteine concentrations are often due to deficiency of folate and also vitamin-B12, as these two vitamins are necessary for the remethylation of homocysteine to methionine. The role of homocysteine in the pathogenesis of diseases associated with hyperhomocysteinemia has been discussed for years, but it remains unclear. On the other hand immune system activation appears to be strongly involved in atherogenesis and also in the pathogenesis of other diseases found to be associated with moderate hyperhomocysteinemia, e.g. elevated concentrations of immune activation markers like neopterin or sTNF-R II have been observed in patients. To study a possible influence of immune stimulation on homocysteine metabolism, in vitro-experiments were performed. Human peripheral blood mononuclear cells (PBMC) were used to monitor the interaction between different immunocompetent cells like T-cells, monocytes/ macrophages and were stimulated with different stimuli. Whereas LPS, TNF- α and Interferon- γ -stimulation did not influence homocysteine production, mitogens concanavalin A (Con A), phytohaemagglutinine A (PHA) and pokeweed mitogen (PWM) significantly increased homocysteine production in comparison with unstimulated PBMC. The dose-dependant increase of homocysteine production induced by Con A and PHA was more expressed than in cells stimulated with PWM. Supplementation of stimulated cells with methionine and folate resulted in altered homocysteine production: Methionine supplementation led to significantly higher homocysteine concentrations, whereas folate supplementation slightly lowered homocysteine accumulation. Interestingly, homocysteine production was also strongly influenced by the anti-inflammatory drug aspirin. Dose-dependently homocysteine production was inhibited, preincubation of cells with 5 mM aspirin before stimulation even resulted in a complete inhibition. Thus, homocysteine production in stimulated proliferating PBMCs can be suppressed effectively by anti-inflammatory treatment in vitro, suggesting that due to its immunosuppressive capacity, also in vivo aspirin may down-regulate formation of homocysteine.

KINETICS OF TRYPTOPHAN DEGRADATION AND NEOPTERIN FORMATION MEASURED IN URINE OF A PATIENT BEFORE AND DURING AN INFECTION EPISODE

Schubert C., Winkler C., Wirleitner B., Noisternig B., Geser W., Fuchs D.

Department of Medical Psychology and Psychotherapy, Institute of Medical Chemistry and Biochemistry, University of Innsbruck, Innsbruck, Austria

Th1-type cytokine interferon-(IFN)- γ induces neopterin production in human monocyte-derived macrophages. In parallel, IFN- γ stimulates indoleamine (2,3)-dioxygenase (IDO) in various cells resulting in the formation of kynurenine and other products at the expense of tryptophan. Thus, in diseases linked with Th1-type immune activation, increased neopterin concentrations and decreased tryptophan levels are common. During a follow-up study of a healthy female during 56 days, twice daily whole amount of urine was collected from 8 a.m. till 8 p.m. and from 8 p.m. till 8 a.m. Urinary specimens were protected from sedimentation and oxidation by adding EDTA plus sodium-hydrogensulfite, and samples were frozen immediately at -20°C until measured. Urinary neopterin concentrations (expressed as μmol neopterin/mol creatinine) were determined as well as tryptophan and kynurenine concentrations using HPLC. Kynurenine per tryptophan ratio (kyn/trp) was calculated as an index for IDO activity. During the observation period the female experienced an infectious episode (around day 47), representing as fever, vomiting and headache, however, not serologically verified. Neopterin concentrations fluctuated within the normal range, and the mean value before day 43 was mean \pm S.D.: $151 \pm 74.6 \mu\text{mol/mol}$ creatinine. Twenty % increase of neopterin concentration was observed at day 46, further increasing on day 47 to reach 147% of baseline. Thereafter a broad peak was developing (6-fold increase from baseline throughout 3-4 days). At the time of infection, also a rapid biphasic increase of kyn/trp was observed reaching approximately 40-fold higher levels than baseline (1345 versus $35.8 + 5.89 \text{ mmol/mol}$ at baseline follow-up), and declining sharply during the following days. Statistical comparison shows on the one hand a significant relationship between neopterin and kyn/trp (all days: $r_s = 0.462$, $p < 0.001$; days 1-43: $r_s = 0.190$, $p < 0.05$; days >43: $r_s = 0.556$, $p < 0.01$). Data indicating activation of IDO being responsible for tryptophan metabolic changes. Time series analysis revealed a significant association between changes of neopterin and of tryptophan metabolism. Specifically the increase of kyn/trp preceded the increase of neopterin by 36 hours, and the increase of neopterin concentrations was followed by a decrease of kyn/trp after 36 hours. Our observations confirm the close relationship between neopterin production and tryptophan degradation. Both metabolic changes can be sensitively detected in urinary specimens which allows easy daily monitoring. The data also indicates an interrelationship between the two metabolic changes: it appears that the immediate decline of tryptophan concentrations during the initial phase of cell-mediated immune response has an impact on the behaviour of neopterin concentrations and vice versa. Clearly, this is an observation based on the study of one person only. Thus, it needs to be confirmed in further individuals.

MYOCARDIAL FUNCTION IN CIRRHOTICS UNDERGOING LIVER TRANSPLANTATION (LT)

Senzolo M., Graziotto A., Bassanello M., Zucchetto P.^o, Maraglino G.[^], Bellotto F.[^], Cillo U., Faggioli S., Burra P., Naccarato R.

Department of Surgical and Gastroenterological Sciences, ^oNuclear Medicine Unit, [^]Cardiology Unit, University of Padua, Padua, Italy

Myocardial perfusion defects asymptomatic in cirrhotics increase the risk of intraoperative cardiac complications during LT.

Aim: to evaluate the myocardial function by using the myocardial perfusion scintigraphy in patients with liver cirrhosis before LT.

Materials and Methods: 30 patients (23 M, 7 F, mean age 49.5, range 27-63 yrs); Child-Pugh B=8, C=22) were studied. The etiology of liver cirrhosis was: alcohol (A) in 5, viral (HBV, HCV, HDV) (V) in 17, mixed (A+V) in 3, cholestatic in 5. Evaluation of coronary risk factors and hypertension, EKG, echocardiography, myocardial perfusion scintigraphy GSPECT (99m TcMIBI) at rest and after dipyridamole infusion were assessed. Coronarography was performed in patients with myocardial perfusion defects.

Results: 30% of patients were diabetics; 40% had smoking habits. All patients had a normal ejection fraction. Rest myocardial imaging and stress perfusion were negative in 27/30 patients. Two patients with positive dipyridamole test underwent coronarography which was negative, the last one died in the waiting list. Twenty-seven patients underwent LT with excellent cardiologic performance both intraoperatively and during the postoperative follow up (range 1-18 months).

Conclusions: Stress-rest myocardial imaging is a non invasive and well tolerated screening test for the detection of coronary artery disease before LT.

SERUM HUMAN PROLYL-HIDROXILASE AND TYPE-IV COLLAGEN IN FOLLOW-UP OF CHILDREN WITH SEVERE CHRONIC LIVER DISORDERS

Tamaro G., Gerarduzzi T., Biasotto E., Torre G.*, Martellosi S.

*Department of Pediatrics, IRCCS Burlo Garofolo, University of Trieste, *Department of Pediatrics, Ospedali Riuniti, Bergamo, Italy*

Objective: To evaluate serum levels of prolyl-hydroxylase and helical domain of Type IV collagen as markers of hepatic fibrogenesis in several pediatric condition related to chronic liver damage.

Design: Prolyl-hydroxylase and Type IV collagen were determined during the follow-up of children affected by severe chronic hepatic disorders.

Methods: 11 patients and 80 healthy controls have been studied. 3 children were affected by sclerosing colangitis in ulcerative colitis, 3 had biliary cirrhosis after Kasai surgery and 1 after liver transplant for biliary atresia, 2 presented autoimmune hepatitis related to celiac disease and 1 after liver transplant for biliary atresia, 1 was affected by Byler disease. Prolyl-hydroxylase and helical domain of Type IV collagen were measured by using immunoenzymatic methods. Other hepatic markers were determined and liver biopsy were performed in 7/11 patients.

Results: In the patients prolyl-hydroxylase (49.6 ± 11.4 ng/ml) was not significantly different from controls (39.1 ± 5.9 ng/ml). On the contrary, the patients showed a mean Type IV collagen (235.6 ± 167.2 ng/ml) significantly ($P < 0.01$) higher than controls (100.2 ± 10.5 ng/ml). A good relationship between liver fibrosis and the Type IV collagen serum level was found, since 6/7 children undergoing biopsy showed various degree of fibrosis and had high level of type IV collagen in at least two serum samples taken before performing the biopsy. Beside some slight increases of aminotransferases, all the other hepatic markers were not significantly altered.

During the study, the patient affected by Byler disease underwent liver transplant because of liver failure. Her serum levels of type IV collagen were impressively high before transplant and decreased significantly until reaching values comparable to controls in 6 months after transplant.

Conclusion: In patients with chronic liver disease, prolylhydroxylase is not a specific marker of hepatic fibrosis, while Type IV collagen is a useful tool for evaluating fibrogenic activity and eventually to determine when to perform follow-up biopsies. Further studies may be interesting in follow-up of patients undergoing liver transplant to better understand the kinetic of these markers.

LONG-TERM PRESERVATION OF RAT LIVER: A COMPARATIVE EVALUATION OF CELSIOR VERSUS UW SOLUTION

Vairetti M.¹, Ferrigno A.¹, Bertone R.², Griffini P.,² Richelmi P.,¹ Freitas I.²

¹Dept. of Internal Medicine and Therapeutics; ²Dept. of Animal Biology and CNR-IGM, University of Pavia; Italy

Orthotopic liver transplantation (OLT) has become a well-established therapy for end-stage liver disease of various etiologies. The University of Wisconsin (UW) solution is the most widely used preservation solution for liver storage. Celsior solution, usually used for heart transplantation, has also been proposed as a cold-storage solution for liver, but its use is still under investigation. Preservation solutions allowing to prolong the time span of preservation and to improve the survival of *all* types of liver cells are still looked for. While in the literature the successful use of UW for 48 hours has been reported, to our knowledge, no studies have been performed to compare Celsior with UW after 48 hr of liver preservation at 4°C. In this study the efficiency of UW and Celsior preservation solution on hepatocellular and endothelial injury was investigated after 48 hr of cold storage in a model of isolated perfused rat liver.

Livers were harvested from male Wistar rats and then flushed with either Celsior or UW solution and stored for 48 hr at 4°C in the respective solution. Reperfusion was performed using a non-recirculating system with oxygenated Krebs-Henseleit buffer at 37°C. After ischemic storage we evaluated, in the effluent perfusate, lactate dehydrogenase (LDH), as an index of hepatocyte damage, hyaluronic acid (HA) uptake, as an index of sinusoidal endothelial cell viability and thiobarbituric acid-reactive substances (TBARS), as index of lipid peroxidation. Tissue reduced and oxidized glutathione (GSH; GSSG), and ATP were also evaluated. Histochemical assays were performed for *in situ* detection of LDH, glycogen and reactive oxygen species (ROS).

Light microscopy of the specimens prepared at the end of reperfusion period from the livers preserved in Celsior or UW showed that the damage was quantitatively and qualitatively more pronounced in the livers preserved in Celsior. Particularly, the hepatocyte damage was higher and parenchyma eosinophilia lower with livers preserved in Celsior than those with UW. During the reperfusion period, which followed the 48 hr of cold storage, significantly more LDH was released in the effluent perfusate in the livers preserved in Celsior than in those stored in UW. *In situ* detection of LDH activity revealed decreased activity after the reperfusion period for both solutions; however, higher levels were demonstrated in the livers stored with UW as compared with Celsior, thus confirming that the damage was more pronounced in the livers preserved with Celsior. There was an immediate marked decrease in HA uptake during reperfusion, which was significantly higher in the livers preserved with Celsior than in those stored with UW.

After the reperfusion period, hepatic levels of ATP decreased in both solutions but were significantly lower in the livers stored with Celsior. The amount of glycogen decreased under both conditions at the end of reoxygenation at 37°C but the levels were higher in UW than in Celsior. At the end of reperfusion, the livers exhibited a significant reduction in GSH levels and in GSH/GSSG ratio. In the liver preserved with UW, the GSH levels and GSH/GSSG ratio were higher than in the livers stored with Celsior. Moreover, lipid peroxidation in the perfusate was lower using UW than Celsior. The analysis of ROS production in periportal hepatocytes and sinusoidal cells revealed quantitatively and qualitatively higher levels of ROS for the livers preserved in Celsior. These data are in keeping with the biochemical findings for GSH, GSH/GSSG ratio, lipid peroxidation and HA uptake.

In conclusion, our data demonstrate that UW is the better solution when the livers are preserved for a long time, in this experimental model. Celsior is probably useful for short cold storage periods but it is not an effective solution for the long-term cold preservation of the liver. However, further *in vivo* experimental studies are required to validate the results obtained with this experimental model. (MIUR-COFIN 2001; FAR 2001).

EXOGENOUS MELATONIN INCREASES BILE PRODUCTION AFTER COLD STORAGE AND REPERFUSION IN RAT LIVER

Vairetti M.¹, Ferrigno A.¹, Bertone R.², Rizzo V.³, Richelmi P.¹, Reiter R.J.⁴, Freitas I.²

¹Dept. of Internal Medicine and Therapeutics, ²Dept. of Animal Biology and CNR-IGM, ³Dep. of Biochemistry, IRCCS Policlinico San Matteo, University of Pavia, Italy. ⁴Department of Cellular and Structural Biology, University of Texas at San Antonio, USA

Melatonin, found at extremely high levels in the bile, is thought to protect bile duct cells from bile toxicity and to prevent oxidative damage to the intestinal epithelium by bile acids (1). It is a powerful scavenger of free radicals at both physiological and pharmacological concentrations, both *in vitro* and *in vivo* (2). Exogenous melatonin showed to increase bile production by preserving the functional and energetic status during warm ischemia/reperfusion associated with reduced concentration of TNF- α and to inhibit the expression of iNOS and NO production (3). The rapid restoration of biliary secretion is an important index of hepatic functional restoration after cold ischemic injury (4).

Although the use of melatonin in the transplantation field was suggested (5) till now it had not been tested in a liver cold preservation and reperfusion model. If storage time exceeds over 10 to 12 hours, late posttransplant complications such as biliary stricture occur in more than 25% of liver transplant recipients (6). Based upon the above premises we used the isolated and perfused rat liver model for studying the effect of melatonin added during reperfusion, as an innovative strategy to control liver damage after hepatic cold preservation.

Livers were harvested from male Wistar rats and then flushed with two different preservation solutions, Celsior or UW, and stored for 20 hr at 4°C in the respective solution. Reperfusion (120 min) was performed using a non-recirculating system with oxygenated Krebs-Henseleit buffer at 37°C without and with glucose 5 mM. In some experiments melatonin 100 μ M was added in the perfusate during the reperfusion period. After ischemic storage we evaluated, in the effluent perfusate, lactate dehydrogenase (LDH), as an index of hepatocyte damage, hyaluronic acid (HA) uptake, as an index of sinusoidal endothelial cell viability and thiobarbituric acid-reactive substances (TBARS). Tissue reduced and oxidized glutathione (GSH; GSSG) were also evaluated. Bile secretion analysis was performed measuring melatonin content and γ -glutamyl transpeptidase (γ -GT) levels.

LDH release was similar during the all reperfusion period using UW or Celsior solution in absence or presence of melatonin. These results were observed also in presence or absence of 5 mM glucose in the perfusate medium. Bile production was higher when melatonin was added during the reperfusion period either with UW and Celsior solutions also in presence or absence of glucose. Release of γ -GT to the bile from cholangiocytes was higher in Celsior than in UW perfused liver; the addition of melatonin to the perfusate reduced Celsior-induced enzyme release. Melatonin had no effect on HA uptake and TBARS formation in the perfusate. In addition, no statistically significant difference of GSH/GSSG ratio was detected. Bile flow is an indicator of cell energy status even after prolonged ischemia and it reflects the ability of the liver to generate ATP during reperfusion (6).

Our preliminary data indicate that exogenous melatonin improves liver function during cold preservation and reperfusion, thus confirming its potential in liver transplantation. Further research to evaluate the mechanisms by which melatonin treatment increases bile production either using UW or Celsior preservation solutions is in progress. (Funds from MIUR-COFIN 2001; FAR 2001/02).

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CLINICAL SIGNIFICANCE OF ALPHA-FETOPROTEIN mRNA IN BLOOD OF HCC PATIENTS WITHOUT EXTRAHEPATIC SPREAD

Vitale A., Bassanello M., Ciarleglio F.A., Basso D.*, Navaglia F.*, Boccagni P., Brolese A., Zanus G., D'amico F., Senzolo M., Bridda A., D'amico D.F., Cillo U.

Dipartimento di Scienze Chirurgiche e Gastroenterologiche "P.G. Cevese"

**Dipartimento di Medicina di Laboratorio, Sezione di Biologia Molecolare, Università di Padova*

Background / Aims: Plasmatic AFP mRNA reflects the presence of circulating HCC cells and it is therefore a sensitive marker of HCC extrahepatic metastases. However, the relationship between AFP mRNA and the main HCC clinical-pathological parameters in patients without macroscopic evidence of extrahepatic spread remains controversial.

Methods: Fifty-one HCC patients were enrolled between April 2001 and October 2002. Forty-four patients (87%) had a cirrhotic liver. Child-Pugh score and liver function tests (transaminase, bilirubin, GGT, albumin) were used to assess the severity of liver disease. HCC diagnosis was confirmed in all patients by percutaneous biopsy or surgical specimen histological examination; pathological grading was assessed in all patients at the same time. AFP levels were also measured in all patients. Tumor characteristics (number and size of lesions, macroscopic vascular invasion, TNM stage) were defined by imaging studies. The status of AFP mRNA in blood was determined when all these HCC clinical-pathological parameters were obtained. AFP mRNA was determined also in 50 patients with diagnosis of cirrhosis (6), colon (24) and pancreatic (20) carcinoma (control group).

Results: AFP mRNA was positive in 20 HCC patients (40%) and in 18 patients without HCC (36%). AFP mRNA did not show any significant correlation with Child-Pugh score, liver function tests, AFP plasma level, number and bilobarity of nodules and TNM staging. On the contrary, the presence of AFP mRNA in blood was significantly related with nodule size ($p=0.03$), vascular invasion ($p=0.006$) and G2-G3 HCC ($p<0.0001$).

Conclusions: Plasmatic AFP mRNA is frequently detected in liver diseases (cirrhosis or potentially metastatic gastroenteric tumors) and HCC. In HCC patients without clinical evidence of extrahepatic metastases AFP mRNA seemed to identify the more biological aggressive tumors.

ANTIOXIDANT RESVERATROL SUPPRESSES CYTOKINE-INDUCED ACTIVATION OF IDO

Wirleitner B., Schröcksnadel K., Winkler C., Frick B., Fuchs D.

Institute of Medical Chemistry and Biochemistry, University of Innsbruck, and Ludwig Boltzmann Institute of AIDS Research, Innsbruck, Austria

Resveratrol is a naturally occurring phytoalexin produced by grapes, being present in the canes, leaves and skins of the berries. Besides red wine, also peanuts contain significant amounts of resveratrol. As phenolic compound, resveratrol contributes to the antioxidant potential of red wine and may thereby play a role in the prevention of human cardiovascular diseases. Further this compound was shown to modulate the metabolism of lipids, and to inhibit the oxidation of low-density lipoproteins and the aggregation of platelets. Resveratrol is thought to be partially responsible for the cholesterol-lowering effects of red wine. Anti-inflammatory and anticancer properties of resveratrol were described. Epidemiologic and clinical studies suggest that consumption of resveratrol-rich foods may result in reduced cardiovascular disease risk, lowered total cholesterol, and lowered LDL cholesterol.

To further investigate the anti-inflammatory effect of resveratrol, we tested a possible interaction between the phytoalexin and cytokine-mediated tryptophan degradation. During cell-mediated (Th₁-type) immune response large amounts of interferon- γ (IFN- γ) are produced. IFN- γ is the main stimulator of indoleamine-2,3 dioxygenase (IDO), the first enzyme in the conversion of the essential amino acid tryptophan to kynurenine. Therefore, *in vivo* decreased tryptophan and increased kynurenine levels are detected during Th₁-immune response. Activation of IDO and thereby removal of tryptophan inhibits growth of various pathogens. Recently, degradation of tryptophan along the kynurenine pathway was also found to reduce T cell activation. Degradation products, for examples kynurenic acid and 3-hydroxyanthranilic acid were reported to induce apoptosis in T cell populations. Onset of IDO was suggested to be a regulatory tool of macrophages and dendritic cells to down-regulate T cell activation. Due to its immunomodulatory properties, we investigated the effect of resveratrol on IDO activation in human peripheral blood mononuclear cells (PBMC).

PBMC were isolated by density centrifugation from healthy blood volunteers and stimulated with resveratrol with or without mitogenic stimulation of the cells. To determine IDO activation, tryptophan as well as kynurenine concentrations in culture supernatants were measured by high performance liquid chromatography (HPLC) and kynurenine/tryptophan ratios were calculated. Our results show that resveratrol in a dose-dependent manner suppresses IDO activation in stimulated human PBMC. This finding corresponds with earlier reports showing a suppression of T cell development by resveratrol and a reduction of cytotoxic T lymphocytes (CTL) and natural killer cell (NK) activity in PBMC. A decreased IDO-activation by resveratrol would result in a decreased production of degradation products along the kynurenine pathway, some of which were shown to act pro-apoptotic and possibly contribute to tissue damage during inflammation. This might be of especial relevance in chronic immune stimulation and therefore also in the prevention of cardiovascular diseases.