Cellular Oxidative Stress and Peroxisomal Enzyme Activities in Pediatric Liver Transplant Patients

Wafa’a Al-Qabandi    Abdullah F. Owayed    Gursev S. Dhaunsi
Department of Pediatrics, Faculty of Medicine, Kuwait University, Jabriya, Kuwait

Key Words
Liver transplant · Peroxisome · Immunosuppressive · Oxidative stress

Abstract
Objectives: In this study, we examined the activities of key peroxisomal enzymes in peripheral blood lymphocytes (PBLs) of pediatric liver transplant patients. Subjects and Methods: Venous blood was drawn from 14 patients aged 5–16 years on FK-506 treatment and 18 healthy subjects for isolation of lymphocytes. β-Oxidation of very long chain fatty acids (VLCFAs) and activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), NADPH oxidase (NOX), catalase and peroxisomal enzyme acyl CoA oxidase (ACO) were measured in cellular homogenates. Levels of malondialdehyde (MDA) were measured as an index of lipid peroxidation. Protein content and mRNA levels of catalase, peroxisomal membrane protein-70 (PMP-70) and ACO were measured using Western blotting and PCR techniques. Results: PBLs isolated from liver transplant patients showed significantly (p<0.01) increased levels (226.9 ± 24.5 μmol/mg protein) of MDA as compared to the levels in controls (162.8 ± 19.6 μmol/mg protein), whereas enzyme activities of SOD and NOX remained unaltered in patients’ cells. Enzyme activities of catalase and GPx were markedly (p < 0.01) decreased in cells isolated from liver transplant patients. ACO activity and β-oxidation of VLCFAs in PBLs from liver transplant patients were however found to be significantly increased by 38 and 52% respectively when compared with controls. Gene expression of PMP-70 and ACO was also significantly increased (p < 0.01) in PBLs of patients. Conclusion: Our results clearly showed that peroxisomal metabolic activities are markedly altered in lymphocytes of liver transplant patients and might contribute to the development of cellular oxidative stress.

Introduction

Peroxisomes are microbodies responsible for some vital cellular functions such as oxidation of very long chain fatty acids (VLCFAs) and biosynthesis of plasmalogens [1, 2]. The importance of metabolic activities associated with peroxisomes has been well known as various peroxisomal diseases are caused by a single or multiple peroxisomal enzyme defects [3]. Biogenesis of peroxisomes in eukaryotic cells is under tight transcriptional control and determines the selective metabolic activities for per-
oxisomes in different tissues and cell types [4]. Peroxisome metabolism gained significance in human health as VLCFAs were reported to be exclusively β-oxidized in peroxisomes and a defective peroxisomal β-oxidation was linked to several peroxisomal diseases including Zellweger syndrome and X-linked adrenoleukodystrophy. Besides their role in degradation or biosynthesis of various metabolites within the cell, peroxisomes have also been reported to participate in the regulation of cellular oxidative stress [5]. Catalase, a key peroxisomal enzyme, is responsible for degradation of hydrogen peroxide within the peroxisomes, thereby contributing significantly to the cellular antioxidant system. Though catalase is present in peroxisomes of eukaryotic cells, peroxisomes have been shown in recent years to possess other antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) [6]. On the other hand, peroxisomes continuously produce hydrogen peroxide, through the action of acyl CoA oxidase (ACO) enzyme during oxidation of fatty acids or amino acids, and superoxide anions through cytochrome p450 enzymes during metabolism of drugs and xenobiotics [7]. An imbalance in the production of reactive oxygen species (ROS) and their degradation within peroxisomes has been shown to contribute significantly to the development of overall cellular oxidative stress under various pathophysiological conditions [8, 9] that may or may not involve proliferation of peroxisomes. Peroxisome proliferation has been well reported to occur in response to certain xenobiotics and particularly in tissues of experimental animals treated with hypolipidemic drugs such as clofibrate. Besides several-fold increases in peroxisome-specific enzymes such as ACO, peroxisome membrane protein-70 (PMP-70) and β-oxidation of VLCFAs, induction of catalase also occurs as part of peroxisome proliferation.

The liver is a highly metabolic organ and peroxisomes and their metabolic activities are maximal in hepatocytes as compared to their number and function in other mammalian tissues/organs. Several studies on humans and experimental animals have shown that peroxisomal enzyme activities get altered in the liver during pathological conditions such as cancer, diabetes and drug toxicity [7, 10]. Liver transplantation is now a widely carried out surgical procedure to restore severely impaired hepatic function during various diseases in children such as hepatitis, biliary atresia and progressive familial intrahepatic cholestasis [11]. Liver transplantation results in profound overall physiological as well as biochemical changes that include altered immunological status of the patients, increased production of cytokines and development of cellular oxidative stress [12]. Liver transplant patients receive immunosuppressive treatment to minimize immunological disturbances, and commonly used immunosuppressive drugs such as Prograf (FK-506) have been shown to regulate cytokine production and also free radical-producing enzymes [13, 14]. Liver transplant-related complications and postoperative immunosuppressive treatment might have an effect on the cellular redox state and peroxisome enzymatic machinery not only in the liver but in other tissues as well, including blood cells. In view of the increasingly recognized importance of peroxisomes in the regulation of cellular oxidative stress, we examined in this study the status of key peroxisomal enzymes in peripheral blood lymphocytes (PBLs) to explore the role of peroxisomes in free radical-induced pathogenesis of liver transplant complications.

Subjects and Methods

Cell culture media, RPMI 1640, DMEM and DMEM-Ham’s F-12, were purchased from GIBCO (Grand Island, N.Y., USA). Trypsin-EDTA, fetal bovine serum and penicillin/streptomycin were procured from Sigma Chemical Company (St. Louis, Mo., USA). Bovine serum albumin was purchased from Calbiochem (La Jolla, Calif., USA) and all other chemicals and reagents were obtained from Sigma (St. Louis, Mo., USA). Cell culture plates and Petri dishes were obtained from Falcon Becton Dickinson (Oxnard, Calif., USA). Antibodies against PMP-70, catalase, ACO and CD-3 were procured from Abcam (Cambridge, Mass., USA).

Subjects

Fourteen pediatric liver transplant patients (table 1) were recruited for this study. Clinical data and blood samples were collected from the patients and eighteen age- and sex-matched healthy controls. Blood samples were drawn in EDTA-containing tubes and used immediately for setup of cell cultures and biochemical assays. All patients included in this study were on immunosuppressive (FK-506) treatment, whereas healthy controls were drug-free.

Cell Culture and Biochemical Assays

Peripheral blood mononuclear cells were isolated from the blood samples of patients and controls and cultured in growth medium (RPMI 1640 containing 10% fetal bovine serum, and penicillin and streptomycin 5 U/ml and 5 μg/ml, respectively) in a humidified atmosphere of 5% CO₂ and 95% air. Pure cultures of lymphocytes were established by culturing the peripheral mononuclear cells for 5–7 days according to a modified method of Haller et al. [15]. Briefly, whole blood was incubated with carbonyl iron (10 mg/ml) for 1 h at 37°C and the monocytes were removed magnetically. Monocyte-depleted blood was then diluted (1:1) with buffered saline and subjected to Ficoll-Isopaque gradient centrifugation and lymphocytes were collected from the interphase and washed with RPMI 1640. After washing, the cells...
were resuspended in RPMI 1640 growth medium, placed into coated culture flasks and cultured at 37°C in a cell culture incubator for 3 days. Cells isolated from liver transplant patients were cultured in the presence of the immunosuppressive drug FK-506 (25 ng/ml). Purity and yield of the isolated nonadherent lymphocytes were determined using cell surface staining techniques. Cultured lymphocytes isolated from patients and healthy control subjects were centrifuged at 4°C and the cell pellet was homogenized in 0.25 M sucrose buffer, pH 7.2. Cell homogenates were then used for various biochemical assays.

**Enzyme Assays**

NADPH oxidase (NOX) activity was measured in cell homogenates at 37°C using lucigenin and NADPH as described previously [16]. Briefly, cell homogenates were added to a reaction mixture containing 50 mM phosphate buffer, pH 7.1, 0.01 mM EDTA and 25 μM lucigenin, and the reaction was started by adding 100 μM NADPH. Chemiluminescence was recorded over a period of 3 min and specific activity was calculated as relative light units emitted/s/mg protein. Catalase activity was assayed using hydrogen peroxide as substrate according to the method described earlier by Baudhuin et al. [17]. Enzymatic activity of GPx was measured by the glutathione reductase-coupled oxidation of NADPH using cumene hydroperoxide as a substrate according to the method of Wendel [18]. One enzyme unit of GPx activity is defined as 1 μmol of NADPH oxidized/min/mg protein. Activity of total SOD in cell homogenates was assayed using a method based on the inhibition of nitro blue tetrazolium dye reduction by SOD [19]. One unit of SOD enzyme activity is defined as the amount of protein required for 50% inhibition of nitro blue tetrazolium reduction.

**β-Oxidation of Lignoceric Acid and ACO Activity**

β-Oxidation of lignoceric acid, a VLCFA, was measured as described earlier [20] with some modifications involving the use of GC-MS (Shimadzu, Japan). Peroxisomal fatty ACO activity was assayed based on the H2O2-dependent oxidation of leucodichlorofluorescein catalyzed by exogenous peroxidase as previously described by Small et al. [21].

**Lipid Peroxidation Assay**

Plasma and total cellular levels of malondialdehyde (MDA), an index of lipid peroxidation, were measured using an assay kit from Calbiochem Inc.

**Western Blot Analysis**

Cell homogenates were prepared and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using Laemmli’s sample buffer. Following SDS-PAGE, proteins were transferred on to nitrocellulose and immunoblotted with antibodies against catalase, ACO, PMP-70 and actin. Protein bands were visualized using horseradish peroxidase-labelled secondary antibody and ECL reagents. The amount of protein in cell homogenates was measured using the Protein Assay Kit from Bio-Rad Inc.

**RNA Isolation and Reverse Transcription**

Total RNA was extracted from cultured lymphocytes with the RNA extraction kit from Clonetech. Isolated RNA was of high quality and was used immediately for synthesis of first-strand cDNA according to protocols from Clonetech’s SMART PCR cDNA synthesis kit.

### Table 1. Features of 14 pediatric liver transplant patients in Kuwait

| Feature                     | n | %  |
|-----------------------------|---|----|
| Gender                      |   |    |
| Male                        | 9 | 64 |
| Female                      | 5 | 36 |
| Age at transplantation      |   |    |
| 5–12 months                 | 6 | 43 |
| 1–2 years                   | 2 | 14 |
| 3–4 years                   | 3 | 21 |
| 4–5 years                   | 2 | 14 |
| >5 years                    | 1 | 7  |
| Primary defect              |   |    |
| Biliary atresia             | 4 | 28 |
| Neonatal hepatitis          | 2 | 14 |
| PFIC                        | 3 | 21 |
| Hepatoblastoma              | 1 | 7  |
| Other                       | 4 | 28 |
| Family history              | 7 | 50 |
| Immunosuppressive treatment | 14| 100|

**PCR Detection of mRNA for ACO, PMP-70 and G3PDH**

Amplification of cDNA obtained from reverse transcription of RNAs from lymphocytes was carried out using Advantage cDNA PCR kit (BD Biosciences Clonetech) and the following primers: ACO, 5’-ACT ATA TTT GGC CAA TTT TGT G-3’ and 5’-TGT GGC AGT GGT TTC CAA GCC-3’; PMP-70, 5’-AAG TTA ACG AGT GCA ATT GGA GC-3’ and 5’-AGC CCA GAA ACA ACC AAG TAG G-3’. Primers for G3PDH were provided by Clonetech. The first strand of cDNA obtained from reverse transcription was denatured for 1 min at 95°C and subjected to PCR with the following parameters: 95°C for 30 s, 58°C or 62°C for 30 s, 68°C for 45 s, 25–30 cycles after denaturing at 95°C for 1 min. PCR products were then analyzed using agarose gel electrophoresis. For statistical analysis of the data, a two-tailed Student t test was employed.

### Results

**Purity and Yield of Lymphocytes**

The purity of lymphocytes isolated from blood samples of liver transplant patients or control healthy subjects was >95% as determined by cell surface staining with lymphocyte-specific marker antibodies anti-CD3, anti-CD22 and anti-CD57. Lymphocyte yield from blood samples of liver transplant patients (68 ± 10%) was not significantly different from healthy control (72 ± 9%) blood samples (p = 0.92).
**Lipid Peroxidation**

MDA levels measured to assess the degree of lipid peroxidation in plasma and cellular homogenates obtained from blood samples of control subjects and liver transplant patients are shown in figure 1. MDA levels were significantly increased ($p < 0.01$) in PBLs isolated from liver transplant patients when compared to those from control subjects, however there was no marked difference in plasma lipid peroxide content of patients and healthy controls.

**SOD and NOX Activities**

Specific activities of superoxide anion-regulating enzymes SOD and NOX measured in homogenates of PBLs isolated from healthy controls and liver transplant patients are shown in figure 2. Levels of the superoxide anion-producing enzyme NOX and the superoxide-neutralizing enzyme SOD were not significantly ($p = 0.86$) altered in lymphocytes of liver transplant patients. Specific enzyme activity of GPx was however significantly ($p < 0.05$) decreased in PBLs of liver transplant patients as compared to cells from control subjects (fig. 2).

**Peroxisomal Enzyme Activities**

The specific enzyme activities of key peroxisomal enzymes, catalase and ACO, in homogenates of PBLs isolated from control subjects and pediatric liver transplant patients are depicted in figure 3. Catalase activity was found to be significantly decreased ($p < 0.01$) to 41.3 ± 2.6 U in cells of liver transplant patients when compared with control values of 58.6 ± 4.1 U, however the activity
of hydrogen peroxide-producing peroxisomal enzyme ACO was markedly increased \( (p < 0.05) \) in patients. The β-oxidation of the VLCFA lignoceric acid (fig. 4) was significantly increased \( (p < 0.01) \) in the cultured lymphocytes isolated from liver transplant patients as compared to the cells from healthy control subjects. PMP-70 content was also significantly increased \( (p < 0.01) \) in cultured lymphocytes of liver transplant patients as compared to the control as shown in figure 4, however catalase protein content was found to remain unaltered in lymphocytes of liver transplant patients. The gene expression of ACO and PMP-70, two peroxisomal markers (fig. 5), was significantly increased \( (p < 0.01) \) in lymphocytes isolated from patients in this study as compared to control lymphocytes.

**Discussion**

It has been well documented that organ transplantation results in various pathophysiological changes, and immunosuppressive therapy is inevitable in liver and/or kidney transplant patients. Development of cellular oxidative stress and its role in pathogenesis has been widely reported in various liver diseases, complications of liver transplants and associated drug treatments [12], and the results of this study suggest that peroxisomes might have a role to play in the development of cellular oxidative stress in liver transplant patients.

It is an intriguing question whether the observed increase in lipid peroxidation in liver transplant patients is due to the sustained ischemia/reperfusion-induced oxidative stress of the transplant procedure, posttransplant complications or the immunosuppressive therapy. Pretransplant ischemia/reperfusion injury as well as liver transplant-related complications have been reported to cause overproduction of cytokines and highly reactive free radical moieties [22]. A role for ROS in mediating hepatic ischemia-reperfusion injury has been well reported with emphasis on the use of antioxidants as therapeutic agents in liver transplant patients [23]. Though the reports on development of oxidative stress during the posttransplant period while patients are also taking immunosuppressive drugs are conflicting, pharmaceutical agents such as tacrolimus that are commonly used in liver transplant patients have been shown to exert antioxidant and anti-inflammatory effects [24, 25]. Enhanced lipid peroxidation in PBLs of our liver transplant patients could be further attributed to deficiency of endogenous antioxidants. Glutathione deficiency and depletion of an-
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Our patients. Liver transplantation and postoperative drug therapy in superoxide-generating enzyme, remained unaffected by oxidation. However, enzyme activity of NOX, a major superoxide-generating enzyme, remained unaffected by liver transplantation and postoperative drug therapy in our patients.

Recent reports [27, 28] have shown that the anti-inflammatory and antioxidant effect of some immunosuppressive compounds is mediated through inhibition of NOX, and it is quite possible that immunosuppressants regulated the NOX activity in tissues of our patients. Our findings that enzyme activities of SOD and NOX were not affected in liver transplant patients suggest that the observed increase in cellular oxidative stress in our patients, as manifested by enhanced levels of lipid peroxides, may not be superoxide anion-mediated. In addition to partial loss of GPx activity, a significantly impaired activity of the peroxisomal marker enzyme catalase in PBLs of our patients suggests a possible role for peroxisomes in the regulation of cellular hydrogen peroxide and the enhanced peroxidation of lipids in cells of liver transplant patients. Enhanced activity of ACO, a key hydrogen peroxide-producing enzyme in peroxisomal metabolism, further underscores a possible contribution of peroxisomes in the development of oxidative stress in tissues of liver transplant patients. β-Oxidation of VLCFAs is an important metabolic function of peroxisomes in human cells and tissues, and its marked increase in lymphocytes of liver transplant patients complements the enhanced activity of ACO and possibly translates into an increased production of hydrogen peroxide. Increased cellular levels of hydrogen peroxide have been shown earlier to inhibit catalase activity [5], and this could be due to the observed decrease of catalase activity in our patients, though the cellular levels of catalase protein were not altered in our patients as compared to the healthy control subjects. A role for peroxisomes in the regulation of cellular oxidative stress has emerged recently through reports demonstrating the modulation of ROS-metabolizing peroxisomal enzymes during various pathophysiological conditions [8, 9]. Our findings in this study indicate for the first time an involvement of peroxisome-mediated cellular oxidative stress in the pathogenesis of liver transplantation. It remains to be investigated whether the observed modulation of peroxisomal activities in transplant patients is actually the result of transplant complications or an effect of posttransplant immunosuppressant drug(s) on the peroxisomal system. A number of reports [29, 30] have illustrated that immunosuppressive agents such as tacrolimus activate peroxisome proliferator-activated receptors (PPARs), however any effect of such pharmacological agents on the peroxisomal enzyme system is not known as yet. The marked increase in gene expression of PMP-70 and ACO accompanied by enhanced β-oxidation of VLCFAs in this study clearly indicates that the immunosuppressive agent FK-506 might induce proliferation of peroxisomes, besides its other effects such as activation of PPARs.

This study clearly illustrated the modulation of peroxisomal metabolic activities at the transcriptional level in lymphocytes of liver transplant patients during treatment, and it is quite possible that the immunosuppressive agent FK-506 might affect the peroxisomal system in other tissues as well. Development of cellular oxidative stress in liver transplant patients is the result of several factors including metabolic activities of mitochondria and cytosol, yet in view of our findings, peroxisomes might be expected to play a significant role in ROS-mediated complications of liver transplantation.

Conclusion

This study demonstrated that liver transplant patients experience a certain degree of cellular oxidative stress despite immunosuppressive therapy and underscored the importance of peroxisomes in the pathogenic mechanisms of liver transplantation and postoperative immunosuppressive therapy.

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