Reduced Expression of the Melanocortin-1 Receptor in Human Liver during Brain Death

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Brain death • Intercellular adhesion molecule-1 • Liver inflammation • Melanocortin-1 receptor

Abstract
Objective: There is evidence that brain death has detrimental effects on peripheral organs. Clinical and experimental studies on organ donors showed marked inflammation in tissue samples of livers and kidneys collected during brain death. The inflammatory reaction is characterized by release of cytokines and inflammatory cell infiltration. Because melanocortins and their receptors are significant modulators of inflammation, we hypothesized that downregulation of melanocortin receptors during brain death could contribute to enhance inflammation. Methods: Using real-time polymerase chain reaction (PCR) analysis, we determined expression of melanocortin receptors in liver biopsies obtained from brain-dead organ donors before cold ischemia and in normal liver tissue during resection of benign focal lesions of the liver. Tissue biopsies were also analyzed for expression of intercellular adhesion molecule-1 (ICAM-1), which has a central function in inflammatory cell migration. Results: Expression of melanocortin-1 receptor (MC1R) mRNA was markedly reduced in liver samples obtained from brain-dead organ donors compared to hepatic tissue collected during resection of benign focal lesions of the liver. Conversely, expression of the adhesion molecule ICAM-1 was significantly increased in livers of brain-dead organ donors.

Conclusions: Disruption of the endogenous anti-inflammatory circuit based on MC1R could contribute to tissue damage during brain death.

Introduction
Studies on organ transplantation indicate that brain death has detrimental effects on donor organs [1]. Indeed, damage caused by brain death in peripheral organs has been demonstrated both in human donors and during experimental induction of brain death in animal studies [2–6]. Tissue biopsies collected during brain death show a marked inflammatory response characterized by release of cytokines and chemokines and by inflammatory cell infiltration [2, 4, 7, 8]. Apoptosis, which could contribute to decreased organ viability and post-transplant organ dysfunction, has also been found [3, 9]. Pre-treatment of the multiorgan donor could be a novel strategy to improve transplantation outcome and to expand the donor pool. This approach would be novel in that it transfers therapeutic intervention from the recipient to the donor.

However, adequate treatment of the organ donor first requires precise recognition of the mechanisms that trigger tissue damage. So far, the main focus in research on brain-death-induced organ damage has been on description of the inflammatory response with little attention to...
its cause. There is now evidence that melanocortin peptides adrenocorticotropic hormone (ACTH) and α-, β-, and γ-melanocyte-stimulating hormone (α-, β-, and γ-MSH, respectively), and their melanocortin receptors are components of a significant endogenous anti-inflammatory circuit [10]. It is clear that integrity of this pathway is very important to maintenance of adequate control of inflammatory reactions [11–17]. We previously found that circulating α-MSH is markedly reduced during severe brain injury [18]. Concomitant downregulation of melanocortin receptors during brain death could contribute to enhance inflammation. It appears important, therefore, to ascertain the expression level of melanocortin receptors in donor livers.

The purpose of this research was to determine gene expression of melanocortin receptors in liver biopsies obtained from brain-dead organ donors before cold ischemia and organ harvest. Control samples were liver specimens obtained during resection of benign focal lesions of the liver. Further, to determine whether brain death was associated with an inflammatory reaction in the liver, we analyzed hepatic tissue for expression of the adhesion molecule ICAM-1, which has a central function in inflammatory cell migration.

**Patients and Methods**

**Subjects and Sampling Procedure**

Liver specimens were obtained from 19 brain-dead organ donors, 9 females and 10 males, mean age 45.2 years (range 21–74). The causes of death were traumatic brain injury (n = 10) and subarachnoid hemorrhage (n = 9). The inclusion criterion was appropriateness for transplantation. Patients with malignancy or hepatic viral infections were excluded. All the livers were transplanted successfully. There was no delayed graft function or primary nonfunction. Needle biopsy (16-gauge) was performed before cold ischemia and organ harvest. Based on the protocol approved by the local Ethical Committee, the tissue portion used for molecular biology analysis was a fraction of the sample obtained for routine histological evaluation. Control samples were specimens obtained during resection of benign focal lesions of the liver in 9 subjects, 4 females and 5 males, mean age 46.8 years (range 22–61), who gave their informed consent.

**RNA Isolation**

Liver specimens weighing 10–50 mg were immediately submerged in 500 μl of 2× Nucleic Acid Purification Lysis Solution (Applied Biosystems, Foster City, Calif., USA). Samples were stored at -80°C until RNA isolation. Tissues were homogenized with an Ultra-Turrax tissue homogenizer (IKA Labortechnik, Staufen, Germany). Total RNA was isolated on an ABI PRISM 6100 Nucleic Acid PrepStation, using Tissue Pre-Filter Tray and Total RNA Chemistry (Applied Biosystems). An RNase-free DNase treatment was performed in order to remove genomic DNA contamination. Finally, RNA was checked for integrity by electrophoresis on denaturing agarose-formaldehyde gels and quantified by optical density measurement (260 nm).

**Real-Time RT-PCR**

Expression of MC1R, MC3R, MC4R, MC5R, and ICAM-1 was evaluated using real-time polymerase chain reaction analysis based on TaqMan technology (Applied Biosystems). First-strand cDNAs (cDNA) were prepared using 1 μg of each RNA sample, 20 pmol random hexamer primers, and 200 U Moloney murine leukemia virus reverse transcriptase (BD Clontech, Palo Alto, Calif., USA) in 20 μl of reaction volume. PCR was performed in an ABI PRISM 7000 sequence detection system (Applied Biosystems) using 4 μl of each diluted (1:5) cDNA in a final volume of 25 μl. The PCR mixture contained 1× TaqMan Universal PCR Master Mix with AmpErase UNG enzyme, and a specific pre-designed 900 nM primer and 250 nM probe mix (Assays-on-Demand Gene Expression Products; Applied Biosystems). Based on the 5’ nuclease chemistry, each assay consists of two unlabeled PCR primers and a 6-carboxyfluorescein-dye-labeled TaqMan minor groove binder probe. Assay IDs were Hs00267167_s1 for MC1R, Hs00252036_s1 for MC3R, Hs00271877_s1 for MC4R, Hs00271882_s1 for MC5R, and Hs00164932_m1 for ICAM-1. Prior to amplification, Amplitaq Gold enzyme was activated by heating for 10 min at 95°C. All genes were amplified by a first step of 15 s at 95°C, followed by a second step of 1 min at 60°C for 50 cycles. Quantitation of specific mRNA targets was normalized for differences in the amount of total RNA added to each reaction using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control, by means of a VIC-labeled TaqMan minor groove binder probe (Pre-Developed Assay Reagent ID 4333764F; Applied Biosystems). Relative quantitation of gene expression was performed using the comparative C_T method (ΔΔC_T). The C_T (threshold cycle) value was defined as the number of PCR cycles required for the fluorescence signal to exceed the threshold value (defined as 10 times the standard deviation of the baseline variation). ΔC_T values were defined as the difference between the C_T of the target mRNA and the C_T of GAPDH mRNA. The x-fold change in each target mRNA was calculated according to the formula 2^{ΔΔC_T}, where ΔΔC_T is the difference between each ΔC_T and the ΔC_T of the sample with the lowest mRNA (calibrator).

**Statistical Analysis**

Values are expressed as means ± SEM. Statistical analysis was performed using Student’s t test. A probability value <0.05 was considered significant.

**Results**

Expression of MC1R mRNA was markedly reduced in liver samples obtained from brain-dead organ donors relative to expression in hepatic tissue collected during resection of benign focal lesions of the liver (fig. 1). Expression of the adhesion molecule ICAM-1 was conversely significantly increased in livers of brain-dead subjects.
There was no expression of the other melanocortin receptors, MC3R, MC4R, and MC5R, in either brain-dead or normal subjects.

Histological examination of livers from brain-dead subjects showed absent (n = 4), mild (n = 10), or moderate (n = 5) inflammatory cell infiltration. The subjects with more pronounced inflammatory cell infiltration tended to have greater ICAM expression; however, as the proportion of subjects with moderate inflammatory cell infiltration was small, no statistically significant correlation could be found. MC1R expression was consistently low across subjects with no clear relation with histological pattern.

Discussion

The data show reduced expression of the melanocortin receptor subtype MC1R in the livers of brain-dead organ donors. Because the melanocortin/melanocortin receptor system has potent anti-inflammatory influences, impaired activity of this endogenous pathway could contribute to enhance inflammation and tissue damage. Indeed, in livers from brain-dead organ donors there was increased expression of the adhesion molecule ICAM-1. These changes may amplify ischemia/reperfusion injury during the transplant procedure and accelerate graft rejection after transplantation.

ACTH and α-, β-, and γ-MSH are proopiomeLANocortin-derived peptides collectively called melanocortins. α-MSH and other melanocortins exert anticytokine and anti-inflammatory effects in blood cells, cells of the immune system, and in other cell types including neural, endothelial, and epithelial cells [10]. α-MSH downregulates the production of proinflammatory cytokines [10] and the expression of costimulatory molecules on antigen-presenting cells [14]. Further, α-MSH has anti-apoptotic effects [19, 20] that could be very helpful to prolong graft survival. Anti-inflammatory effects of melanocortins are mediated, in some part, through inhibition of activation of the nuclear transcription factor NF-κB [21, 22].

Recognition and cloning of melanocortin receptors has greatly improved understanding of melanocortin/target cell interactions [23]. Five melanocortin receptors (MC1R–MC5R) are currently recognized [24, 25]. They are small G-protein-linked receptors with seven transmembrane domains. MCRs are highly conserved in mammals and differ in their tissue distribution and affinity for the various melanocortins. The transmembrane signaling involves stimulation of adenyl cyclase followed by cAMP-induced activation of protein kinase(s) and protein phosphorylation [13].

MC1R is the receptor with the highest affinity for α-MSH (α-MSH ≥ ACTH > β-MSH ≫ γ-MSH). Virtually all the cells responsive to the anti-inflammatory effects of melanocortins express MC1R. This receptor occurs in macrophages [26, 27] microvascular endothelial cells [28], neutrophils [29], mast cells [30] fibroblasts [31], and dendritic cells [32]. Thus, this melanocortin receptor subtype likely participates in the anti-inflammatory effects of melanocortin peptides.

Experiments on immunoneutralization of MC1R in the human mononcytic cell line THP-1 provide further support to the idea that MC1R is significant in immunomodulatory effects of α-MSH [33]. MC1R neutralization with a specific antibody increased basal and lipopolysaccharide-stimulated production of tumor necrosis factor-α by THP-1 cells [33]. Similarly, immunoneutralization of MC1R in mesothelioma cells induced expression of interleukin-8, interleukin-6, and transforming growth factor-β [34]. ICAM-1 expression on inflammatory cells...
is under inhibitory control of α-MSH in several conditions, including experimental heart transplantation and renal injury [35, 36]. Therefore, reduced expression of MC1R in donor organs during brain death likely contributes to enhance production of this inflammatory mediator. Indeed, consistent with previous observations [37, 38], we found a significant increase in ICAM-1 expression in liver donors.

The mechanism responsible for MC1R downregulation during brain death is uncertain. We previously found marked reduction of circulating α-MSH in patients with severe brain injury [18]. Because MC1R expression is induced by α-MSH [39] it may be that receptor decrease is linked to reduction of its ligand. However, neural influences or effects exerted by other circulating mediators cannot be excluded. Of interest, acute ACTH administration in brain-dead potential organ donors induced impaired cortisol release [40]. These results are consistent with downregulation of the ACTH receptor MC2R in the adrenal glands, i.e another peripheral melanocortin receptor.

It is now clear that brain death of the donor is an important risk factor influencing graft outcome [1]. Studies on kidney transplantation indicate improved survival of organs derived from living donors over those from brain-dead donors [41, 42]. In addition to other, nonspecific injuries such as organ procurement, preservation, and consequences of ischemia/reperfusion injury, brain death enhances graft immunogenicity and increases host allo-responsiveness. Indeed, brain death triggers a cascade of molecular and cellular events that eventually may contribute to reduce graft function and/or survival. Consequently, novel strategies focused on treatment of the multiorgan donor prior to transplantation could improve organ quality and graft function.

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