Molecular Genetics and Metabolism 109 (2013) 132–138

Contents lists available at SciVerse ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Improved Amino Acid, Bioenergetic Metabolite and Neurotransmitter Profiles following Human Amnion Epithelial Cell Transplant in Intermediate Maple Syrup Urine Disease Mice

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ARTICLE INFO

Article history:
Received 6 January 2013
Received in revised form 18 February 2013
Accepted 18 February 2013
Available online 26 February 2013

Keywords:
Amnion
Transplant
Epithelium
Metabolic
Liver

ABSTRACT

Orthotopic liver transplant (OLT) significantly improves patient outcomes in maple syrup urine disease (MSUD; OMIM: 248600), yet organ shortages point to the need for alternative therapies. Hepatocyte transplantation has shown both clinical and preclinical efficacy as an intervention for metabolic liver diseases, yet the availability of suitable livers for hepatocyte isolation is also limited. Conversely, human amnion epithelial cells (hAEC) may have utility as a hepatocyte substitute, and they share many of the characteristics of pluripotent embryonic stem cells while lacking their safety and ethical concerns. We reported that like hepatocytes, transplantation of hAEC significantly improved survival and lifespan, normalized body weight, and significantly improved branched-chain amino acid (BCAA) levels in sera and brain in a transgenic murine model of intermediate maple syrup urine disease (*imsud*). In the current report, we detail the neural and peripheral metabolic improvements associated with hAEC transplant in *imsud* mice, including amino acids associated with bioenergetics, the urea cycle, as well as the neurotransmitter systems for serotonin, dopamine, and gamma-aminobutyric acid (GABA). This stem cell therapy results in significant global correction of the metabolic profile that characterizes the disease, both in the periphery and the central nervous system, the target organ for toxicity in iMSUD. The significant correction of the disease phenotype, coupled with the theoretical benefits of hAEC, particularly their lack of immunogenicity and tumorigenicity, suggests that human amnion epithelial cells deserve serious consideration for clinical application to treat metabolic liver diseases.

1. Introduction

MSUD (OMIM: 248600) is an inborn error of metabolism characterized by accumulation of BCKAs and BCAAs (leucine, isoleucine, valine) [1]. MSUD results from mutation in any of the 4 subunits (E1α, E1β, E2, E3) of the BCKDH (EC: 1.2.4.4) enzyme complex [1]. All states in the United States perform newborn screening for MSUD. Lifelong dietary restriction is the most common treatment [2], yet compliance can be challenging with the potential for catabolic crisis and irreversible neurological deterioration when the diet is not closely followed.

OLT improves MSUD patient outcome [3], yet this is a highly invasive and expensive procedure and requires lifelong immunosuppression. The primary limitation to OLT is shortage of organs and the necessity for timely availability of suitable livers. Accordingly, hepatocyte transplantation has been investigated as an alternative to OLT for several inherited metabolic diseases. Compared to OLT, cell transplant is advantageous with respect to cost, fewer complications, and the fact that loss of a graft is not immediately life-threatening [4,5]. Murine models of intermediate MSUD (*imsud*) and phenylketonuria (Pah^{enu2}) demonstrated significant improvements following allogeneic hepatocyte transplant,

Abbreviations: 3-MT, 3-methoxytyramine; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin, 5-hydroxytryptamine; ACoA, acetyl-CoA; α-KG, alpha-ketoglutarate; ANOVA, analysis of variance; AEC, amnion epithelial cells; BCAA, branched chain amino acid; BCKA, branched chain keto acid; BCKDH, branched-chain α-ketoacid dehydrogenase; DA, dopamine; DOL, days of life; DOPAC, 3,4-dihydroxyphenylacetic acid; GABA, gamma-aminobutyric acid; hAEC, human amnion epithelial cells; HPLC, high performance liquid chromatography; HVA, homovanillic acid; iMSUD, intermediate maple syrup urine disease; *imsud*, iMSUD mouse model; MSUD, maple syrup urine disease; OAA, oxalacetate; OLT, orthotopic liver transplant; PAH^{enu2}, phenylketonuria mouse model; Tx, transplant; WT, wild type.

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1096-7192/$ – see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.ymgme.2013.02.011

despite low levels of cell engraftment [6–8]. Clinical hepatocyte transplantation has shown efficacy in other metabolic liver diseases, such as Crigler–Najjar type 1, ornithine transcarbamylase, glycogen storage disease, citrullinemia, and others [4,9–15]. Nonetheless, the availability of suitable donor livers for hepatocyte isolation remains one of the primary stumbling blocks in the utility of hepatocytes in clinical transplantation.

Alternatives to hepatocytes, such as stem cells, could help relieve the cell and organ shortages needed for transplants. hAEC are isolated from the epithelial layer of human amnion derived from full term placenta following live birth [16] providing an abundant cell source unfettered by ethical considerations. Amnion membrane and AECs are not immunogenic, and have documented anti-inflammatory, anti-viral, anti-microbial, and anti-fibrotic properties [17]. Importantly, AECs do not express telomerase, are not immortal, and are not tumorigenic when transplanted [18–20]. Similar to pluripotent stem cells, AECs express surface markers (e.g. SSEA-3 & 4, TRA 1–60 & 1–81) and molecular markers (e.g. OCT-4, Nanog, SOX-2,) in culture [18,19,21] suggestive of pluripotency, and AECs differentiate *in vitro* into cells with properties of all three germ layers [19]. Moreover, feeder layers are not required to maintain the stem cell phenotype.

The clinical application of amniotic membranes without immunosuppression has been performed for more than a century without adverse effects [22,23]. Clinical transplant of human AECs, without immunosuppression, to patients with lysosomal storage diseases has shown no evidence of immunogenicity or tumorigenicity [20,24–26]. Recent reports demonstrated methods to differentiate hAEC along a hepatic lineage [27,28] and also reported that freshly isolated, undifferentiated hAEC engraft into mouse liver to adopt a hepatic morphology and expressed mature liver genes at a level similar to adult human liver [27,28].

Based on the considerations introduced above, to determine if hAEC can perform and sustain mature liver functions *in vivo*, and if their transplantation could be useful to treat metabolic liver diseases, we investigated the efficacy of hAEC Tx in a preclinical study with a mouse model of *imsud* [29]. That study demonstrated that the engraftment of undifferentiated hAECs into neonatal *imsud* livers could increase liver BCKDH enzyme activity, thereby transforming this model from a neonatal-lethal disorder to one in which mice survived long-term, recovered somatic growth, and manifested normalization of brain BCAA imbalances [29]. The current report extends our earlier findings, highlights a number of bioenergetic and neurotransmitter improvements in *imsud* mice treated with hAEC transplant, and addresses the question as to whether liver stem cell transplant could improve or correct symptoms and metabolic disturbances measured in extrahepatic tissues. If successful, this therapy may also be useful in correcting similar disturbances in other inborn errors of metabolism.

2. Materials and methods

All animal studies were performed under the University of Pittsburgh Institutional Animal Care and Use Committee approved protocol 0905698.

2.1. Line

A *imsud* mice [6,7,29,36] and the genotyping protocol [37] have been previously described. Animals were provided standard (22% protein) mouse diet.

2.2. hAEC isolation

AEC isolation was performed as described [16] under IRB approved protocol PRO11060413 for use of human placental tissues through the University of Pittsburgh and Magee-Women's Hospital of UPMC.

2.3. hAEC transplant

Methodological details for hAEC transplant were recently described [29].

2.4. Mouse sacrifice and tissue collection

Mice were sacrificed by cervical dislocation when animals appeared moribund, or at the designated end of the experiment (35 or 100 DOL). Immediately prior to sacrifice, whole blood was collected from the facial/submandibular veins using a 5 mm Goldenrod animal lancet (MEDIpoint, Mineola, NY). Serum was isolated using BD Microtainer serum separator tubes (BD #365959, Franklin Lakes, NJ). Immediately following sacrifice the skull was rapidly accessed and the brain was sagittally resected into halves, and individually flash frozen in liquid N_2. Serum and brains were stored at −80 °C until analysis.

2.5. Serum amino acids

Amino acids from mouse serum (*imsud*, *imsud*-hAEC Tx, WT control) were quantified using standard ion-exchange HPLC with postcolumn ninhydrin derivatization. Data were presented as the mean ± SEM and analyzed by one-way ANOVA and post-hoc Tukey's test. To achieve "correction" or "normalization" via hAEC-Tx, three criteria required fulfillment: 1) the value for *imsud* animals must have differed significantly from WT animals; 2) the value for *imsud*-hAEC Tx animals must have differed significantly from the same value for *imsud* animals; and 3) there was no significant difference between values for *imsud*-hAEC Tx and WT animals. "Partial correction" was achieved when criteria 1 and 3 were met.

2.6. Brain amino acids and monoamines

One half-brain was homogenized in ice-cold SeraPrep (Pickering Laboratories, Mountain View, CA), centrifuged, and the resulting supernatant utilized for quantitative amino acid analysis as described above [38]. The other half-brain was homogenized in ice-cold perchloric acid, followed by centrifugation, and the resulting brain extract was utilized for quantitative determination of monoamines (dopamine, serotonin and associated intermediates/end-products) employing reversed-phase HPLC analysis with electrochemical detection [39]. Monoamine data were presented as mean ± SEM and compiled with the Prizm Graph 5.0 program with statistical evaluation as described above. "Correction" or "partial correction" was determined as outlined above.

3. Results

Neurotransmitter and bioenergetic alterations are characteristic of MSUD associated with toxic accumulation of BCAAs [6,30,31], and we therefore comprehensively analyzed biochemical markers in brain and blood as a function of transplant. All changes were generally maintained across time points (two-tailed "*t*"-test) and there were no differences with respect to genotype for any physiological amino acid not discussed here or in our previous report [29].

3.1. Normalization of Krebs and urea cycle amino acids, and sphingomyelin precursors, in whole brain following hAEC transplant at 100 DOL

The urea cycle intermediates citrulline and ornithine were normalized, and arginine was improved >35% to a concentration consistent with that found in WT (Fig. 1A). The amino acids threonine, proline, and lysine, which can all be catabolized into Krebs cycle intermediate end products (pyruvate and succinyl coA, alpha-ketoglutarate (α-KG), and acetyl-CoA, respectively), were also corrected (Fig. 1B). Additionally, phosphoethanolamine and its precursor ethanolamine, pivotal for sphingomyelin formation, were both normalized (Fig. 1C).