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博 士 論 文 概 要

論 文 題 目

Hepatogenic Differentiation
of
Human Mesenchymal Stem Cells from Adipose Tissue:
Potential for Treatment of Liver Disease

ヒト脂肪組織に由来する間葉系幹細胞の
成熟肝細胞への分化誘導法の確立と治療への応用

申 請 者

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Adult stem cells are a “reservoir” of potential cells at various stages of development and can be used for the restoration and regeneration of damaged tissues and organs. Under proper conditions, stem cells may differentiate into specialized tissues and organs. These unique features make them a promising tool for studies on therapy for diseases such as chronic liver disease, heart attack, spinal injuries, stroke, diabetes mellitus and others.

The liver is one target for which the development of stem cell-based therapy is of great significance. Even though an injured liver is highly regenerative, many debilitating diseases lead to hepatocyte dysfunction and organ failure. Treatments such as resection are not applied because of too little remaining liver function. Liver transplantation is the only effective treatment for severe liver injuries. However, because of organ rejection and the limited number of donors, alternative therapeutic approaches are needed. Stem cells could offer a potentially unlimited and minimally invasive source of cells for hepatocyte replacement and liver regeneration.

Mesenchymal stem cells (MSCs) have been found in bone marrow, amniotic fluid, placenta, adipose tissue, umbilical cord blood, and many fetal tissues and organs. Even though isolated from different tissues, they share a similar surface marker profile and differentiation ability.

The hepatogenic differentiation capacity of MSCs has been confirmed in many independent studies. The possibility for their future application in the therapy of liver diseases is very promising. MSCs can be easily obtained from a patient’s own tissues, isolated *ex vivo*, expanded, differentiated toward hepatocytes, and transplanted back into the patient in form of either undifferentiated MSCs or MSC-derived hepatocytes. In the future, together with the development of tissue engineering technologies, MSC-derived hepatocytes may be transplanted back to the patients in the form of three dimensional culture, or liver devices. Such a possibility sidesteps the limits regarding ethical issues and immunocompatibility problems. Importantly, MSCs represent an advantageous cell type for allogenic transplantation as well, since they are immuno-privileged with low MHC I (HLA I) and no MHC II (HLA II) expression, therefore reducing the risk of allogenic transplant rejection. Currently, attention is being given to adipose tissue (AT) as a source of MSCs for regenerative medicine. AT-MSCs are very similar to bone marrow (BM) - MSCs with respect to surface markers profile and differentiation potential. From adipose tissue, a sufficient number of stem cells for stem cell-based therapy may be obtained without invasiveness or damage to a patient’s health.

The studies are a tightly connected trilogy. The content of it describes characterization of AT-MSCs (Part I), their *in vitro* hepatogenic differentiation potentiality, including functionality and cytochrome P-450 activity (Part II), and finally, their therapeutic potential after transplantation into immunodeficient mice with liver injury (Part III). All of the studies have important impact for future establishment of the therapy for liver diseases.

Part I: Characterization of AT-MSCs.

AT-MSCs were isolated from subcutaneous adipose tissue of six gastric cancers undergoing gastrectomy in the International Medical Center of Japan. The AT-MSCs were characterized regarding their surface marker profile by using RT-PCR analyses, immunofluorostaining and flow cytometry. They were sorted by using CD105 conjugated magnetic beads (MACS). CD105 (endoglin) is one of several candidate mesenchymal stem cell markers, and is a receptor for the pleiotropic TGF-β superfamily. An elevated

potential capacity of CD105+ AT-MSCs has been confirmed by differentiation toward mesoderm lineages such as adipo-, chondro-, and osteo-genic.

Part II: *In vitro* hepatogenic differentiation of CD105+ AT-MSCs. Functional analyses of CD105+ AT-MSC-derived hepatocyte-like cells.

Maintenance of a primary human hepatocyte function *in vitro* is difficult if not nearly impossible. Therefore, an alternative source is needed. AT-MSC-derived hepatocyte-like cells can offer an unlimited source of cells for hepatocyte replacement and studies on drug metabolism and toxicology.

The hepatogenic differentiation of CD105+ AT-MSCs was established based on our previous studies on mouse ES cells. It takes five weeks, and is composed of a 3-step-induction system on collagen type I coated dishes: HGF + FGF1 + FGF4 (Step 1), OsM + DEX (Step2), and hepatocyte culture medium alone (Step 3). Hepatocyte-specific characteristics of CD105+ AT-MSC-derived hepatocyte-like cells were evaluated by morphological changes, by the presence of hepatocyte-specific markers (albumin, alpha-fetoprotein, tryptophan 2,3-dioxygenase, hepatocyte nuclear factor-4alpha, and others), by immunochemistry (CYP3A4, transthyretin, cytokeratin 18), and by biochemical assays (LDL uptake, glycogen storage ability, albumin production ability, ammonia detoxification ability). Additionally, the metabolizing activity of CD105+ AT-MSC-derived hepatocyte-like cells was examined. The results include the expression (RT-PCR, western blot, microarray) and the activity (LC-MS/MS) of selected xenobiotic metabolizing enzymes: cytochrome P450s (CYPs) in CD105+ AT-MSC-derived hepatocyte-like cells. The activity of six types of CYP (1A2, 2B6, 2C9, 2C19, 2D6, and 3A) in CD105+ AT-MSC-derived hepatocyte-like cells was detected and compared with the activity of primary human hepatocytes.

CD105+ AT-MSC-derived hepatocyte-like cells exhibited morphology and functions similar to normal human hepatocytes. In summary, using specific growth factors and collagen type I coating, we generated *in vitro* functional hepatocyte-like cells from CD105+ AT-MSCs. The ability of CD105+ AT-MSC-derived hepatocyte-like cells to metabolize xenobiotics makes them an attractive tool for further studies on new drug metabolism and toxicity.

Part III: Modification of hepatocyte induction system. *In vivo* therapeutic potential of unfractionated AT-MSCs and AT-MSC-derived hepatocyte-like cells after transplantation into mice with liver injury.

This last part concerns *in vivo* transplantation. Our study was performed in order to evaluate the possibility of future clinical usage of AT-MSCs in the treatment of liver diseases. Future safety issues for patients require a special approach, such as shortening as much possible *ex vivo* modifications and a hepatogenic differentiation strategy. For that, MACS sorting was not applied and the hepatogenic induction strategy was shortened and modified. At present, it takes two weeks, and it is enriched in pre-treatment with Activin A and FGF4 (one of the cues secreted by septum transversum mesenchyme (STM) and cardiogenic mesoderm at the early stage of endoderm development *in vivo*). Additionally, nicotinamide, insulin-transferrin-selenium, and DMSO were added. After two weeks of this modified induction strategy, AT-MSCs differentiated into functional hepatocyte-like cells. For cell transplantation, undifferentiated AT-MSCs and AT-MSC-derived hepatocyte-like cells were harvested from the dishes and transplanted into mice with liver injury.

Twenty-four hours post CCl_4 injury, undifferentiated AT-MSCs (n=6) (Group III)

or AT-MSC-derived hepatocyte-like cells (n=8) (Group IV) were transplanted intravenously. The results were compared with two controls, mice with liver injury and no cell transplantation (n=3) (Group II), and mice without injury and no cell transplantation (n=3) (Group I). Twenty-four hours after cell transplantation, the mice were sacrificed. The livers were fixed and serial sections were stained with anti-human specific albumin antibodies. The presence of human-specific albumin cells was detected after transplantation with AT-MSC-derived hepatocyte-like cells (Group IV). Blood plasma was evaluated for biochemical parameters, such as ammonia concentration and markers of liver injury: GPT (glutamic-pyruvic transaminase), GOT (glutamic-oxaloacetic transaminase) and UA (uric acid). The results show significant improvement of those factors after transplantation of AT-MSC-derived hepatocyte-like cells (Group IV). Noteworthy is that mice transplanted with undifferentiated AT-MSCs (Group III) surprisingly also revealed improvement of biochemical parameters. Additionally, we found few HLA-1-positive cells after transplantation of AT-MSCs (Group III). A histological examination revealed that AT-MSC-derived hepatocyte-like cells (Group IV) significantly and AT-MSCs (Group III) not significantly improved the morphology of host mice hepatocytes. The livers of mice transplanted with either AT-MSCs (Group III) or AT-MSC-derived hepatocyte-like cells (Group IV), revealed less vacuolar degeneration caused by dilatation of mitochondria and rough endoplasmic reticulum.

We postulate that the involvement of undifferentiated AT-MSCs may be due to their pleiotrophic contribution through their indirect activity. Thus, we analyzed the production of cytokines/growth factors by undifferentiated AT-MSCs, and compared it with bone marrow-derived mesenchymal stem cells (BM-MSCs) and normal human dermal fibroblasts (NHDFs). A higher secretion of IL-1RA, IL-6, IL-8, G-CSF, GM-CSF, MCP-1, NGF, and HGF by AT-MSCs, as opposed to BM-MSCs and NHDFs was detected. An *in vitro* cytokine/growth factor assay demonstrated a higher production of bioactive factors in AT-MSCs than in BM-MSCs and therefore possibly higher trophic activity of AT-MSCs versus BM-MSCs.

The usage of autologous undifferentiated AT-MSCs would be an extremely cheap, fast and hopeful strategy for patients with severe liver diseases. It would eliminate a long waiting time, risk of rejection, and the additional high costs of *ex vivo* manipulations. The safety issues, however, including post-transplantation tumorigenesis, should be carefully evaluated.

reasonable.

Conclusions:

AT-MSCs reveal a capacity to differentiate *in vitro* into functional hepatocyte-like cells, and therefore may be used in future studies on drug metabolism and toxicology. Generated *in vitro* hepatocyte-like cells as well as undifferentiated AT-MSCs *per se* have therapeutic capacity and improve liver function and host hepatocyte-morphology. The usage of autologous AT-MSCs would be an extremely cheap, fast and hopeful strategy for patients with severe liver diseases. The safety issues, however, including post-transplantation tumorigenesis, should be carefully evaluated.

We propose that AT-MSCs, as a highly available source of stem cells, which can be obtained without invasiveness from a patient's own adipose tissue, can be an ideal tool for the future establishment of a new, alternative and sufficient stem cell-based therapy for liver diseases.