Fetal hepatocyte-derived culture medium elicits adipocyte differentiation to bile duct cell lineages in a mouse model

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Abstract. Fetal cells in the developmental stages function with a distinct mechanism in comparison to adult tissues, which may be a useful source for regenerative medicine in postnatal medicine; however, the precise molecular mechanism remains to be elucidated fully. The present study investigated murine fetal hepatocytes, which were cultured *in vitro*, and the supernatants were used for the culture with murine adipose tissue-derived cells. Notably, the results indicated that fetal hepatocyte-derived culture medium elicits the induction of differentiation of adipose tissue-derived cells to bile duct cell lineages, but not to hepatocyte lineages in mice. This indicates that fetal cells possess the multi potentials, which are already absent in adults, and may be useful for regenerative medicine in future.

Introduction

Adipose-derived mesenchymal stem cells (ADSCs) have the potential to differentiate into numerous types of cells including adipocyte, chondrocytes, osteocytes, cardiomyocytes, vascular endotherial cells, pancreatic β cells and hepatocyte cells (1-9). ADSCs can be obtained in high yield with minimal discomfort under local anesthesia (10,11). Therefore, ADSCs are considered a useful source for regenerative therapy (12). However, in particular endodermal types of cells, the differentiation method has not been well established and differentiation efficiency is extremely low. Thus, the development of an efficient differentiation method is extremely important. Several studies suggested that conditioned medium contains several undefined factors (8,12). These undefined factors induce the stem cells to certain specific types of cells (1).

In the present study, ADSCs were cultured in hepatocyte-derived conditioned medium from mice of various ages and conditioned medium from hepatocyte cells. To the best of our knowledge, this is the first study to assess the differentiation of ADSCs into bile cell lineages using murine fetal hepatocyte-derived culture medium.

Materials and methods

Ethical statement. Animal studies were conducted in strict accordance with the principles and procedures approved by the Committee on the Ethics of Animal Experiments of Osaka University (Osaka, Japan).

Isolation of mouse adipose-derived stem cells. Mouse adipose tissue was obtained from 8 adult (8-12 weeks) C57BL/6JJcI mice. (Nihon Clea, Tokyo, Japan). Bilateral inguinal subcutaneous fat pad was removed, minced into sections, collected with ADSC-culture medium, centrifuged at 1,500 rpm for 5 min to remove cell debris, incubated in 0.1% collagenase type IV (Worthington Biochemical Corp., Lakewood, NJ, USA) and agitated in a water bath at 37˚C for 30 min. Subsequently, the mixture was added to ADSC-culture medium and centrifuged at 300 x g for 5 min to remove cell debris. The cell pellets were suspended in ASDS-culture medium and were plated at 500,000/ml following filtration through a 70-μm cell strainer (Corning, Inc., Corning, NY, USA). The cells were cultured at 37˚C and 5% CO_2. At 100% confluence, the cells were split. Culture media were replaced every 2 days. The ADSCs until the fourth passage were used for hepatic differentiation. The ADSC-culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) containing high glucose (Nacalai Tesque, Inc., Kyoto, Japan) with 10% fetal bovine serum (FBS) and 500 μg/ml of penicillin-streptomycin.

Isolation of mouse hepatocytes and preparation of conditioned medium. Mouse hepatocytes were isolated from E13.5, E15.5, E17.5 and E19.5 C57BL/6JJcI mice (Charles River Laboratories, Willmington, MA, USA), either newborn (within 2 weeks) or

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adult (12 weeks) C57BL/6JJcI mouse (Nihon Clea). Briefly, when the mice were sacrificed, the liver was removed, minced into small sections, collected with hepatocyte-culture medium, centrifuged at 85 x g for 3 min to remove cell debris, incubated with 0.1% collagenase type IV (Worthington Biochemical Corp.,) and agitated in a water bath at 37˚C for 30 min. Subsequently, the mixtures were added to hepatocyte culture medium followed by centrifugation at 800 rpm for 3 min to remove cell debris, damaged cells and non-parenchymal cells. The remaining liver parenchyma was collected in hepatocyte culture medium, passed through a 70-μm sterile filter (Corning Inc.) and cultured at 500,000/ml at 37˚C and 5% CO_2. The hepatocyte culture medium consisted of DMEM-high glucose with 10% FBS, 500 μg/ml of penicillin-streptomycin, 0.5 μg/ml insulin (Sigma-Aldrich, St. Louis, MO, USA), 1 μM dexamethasone (Sigma-Aldrich), 10 ng/ml epidermal growth factor (Peprotech, Inc., Rocky Hill, NJ, USA) and 200 μM ascorbic acid (Sigma-Aldrich). Culture media were replaced every 2 days. The conditioned medium was generated according to the previous study by Kawamoto *et al* (12). In detail, these hepatocytes grew to ≤50% confluence with hepatocyte culture medium. Subsequently, the hepatocyte culture medium was replaced with fresh medium on day 2. After a 48-h culture period, the medium was collected (#1) and replaced with fresh medium. Subsequently, CMs were collected every 48 h incubation (#2). These conditioned media (#1 and #2) were pooled and filtered using a bottle-top filter (Corning, Inc.) to remove cells and debris. Conditioned medium samples were frozen at -20˚C for later use. Induction of hepatogenic differentiation of mouse ADSCs was by hepatocyte-conditioned medium. For evaluation of the hepatogenic differentiation ability, mouse ADSCs were cultured with these types of hepatocyte-conditioned medium at 37˚C and 5% CO_2. These cells were maintained by media exchange every 2-3 days for 2 and 4 weeks, and subsequently they were collected for RNA isolation.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from samples using TRIzoL Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), following the manufacturer's protocol, and was treated with ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan) to generate cDNA. Subsequently, PCR amplification was performed for mouse hepatocyte nuclear factor 4, α (*HNf4α*), α-fetoprotein (*Afp*), glucose-6-phosphatase (*G6p*), albumin (*Alb*), cytokeratin 19 (*Ck19*), *Ck7* and sex-determining region-Y-box 9 (*Sox9*). The RT-PCR products ware analyzed by 1% agarose gel electrophoresis and visualized with ethidium bromide.

Results

Differentiation of ADSCs. To investigate the effect of conditioned medium from mouse hepatocyte, ADSCs were cultured using the conditioned medium (Fig. 1). Subsequently the gene expression pattern was analyzed. The hepatocyte marker genes *Hnf4α*, *Afp* and *G6P* were not expressed in undifferentiated and conditioned medium-treated ADSCs (Fig. 2). Alb expression can be observed at an extremely low level in adult hepatocyte-derived conditioned medium-treated ADSCs (Fig. 2). These data suggested that the conditioned medium from mouse hepatocyte could not induce mouse ADSCs to

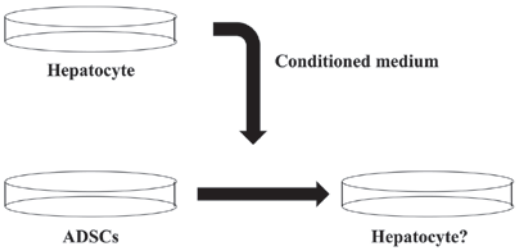

Figure 1. Overview of the experimental design. ADSCs were cultured in hepatocyte-derived conditioned medium to differentiate into hepatocytes. ADSCs, adipose-derived stem cells.

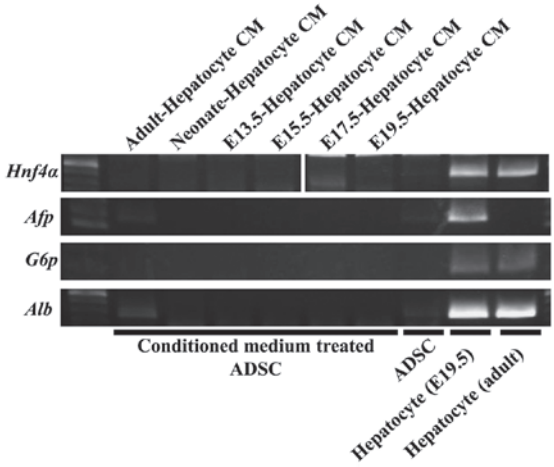

Figure 2. Polymerase chain reaction analysis of hepatocyte marker genes. Gene expression analysis of *Hnf4α*, *Afp*, *G6p* and *Alb*. *Hnf4α*, hepatocyte nuclear factor 4, *α*; *Afp*, *α*-fetoprotein; *G6p*, glucose-6-phosphatase; *Alb*, albumin; ADSCs, adipose-derived stem cells; CM, conditioned medium.

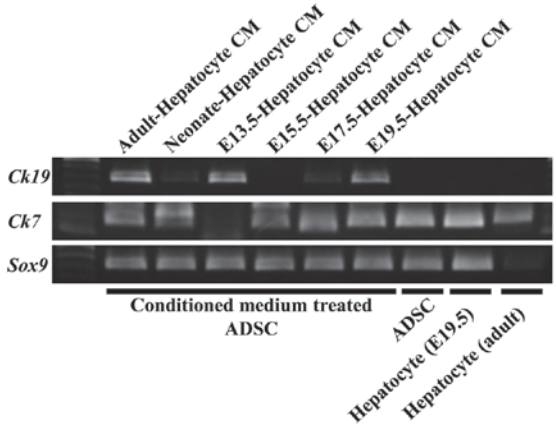

Figure 3. Polymerase chain reaction analysis of bile cells marker genes. Gene expression analysis of *Ck19*, *Ck7* and *Sox9*. *Ck19*, cytokeratin 19; *Sox9*, sex-determining region-Y-box 9; ADSCs, adipose-derived stem cells; CM, conditioned medium.

hepatocyte. By contrast, bile cells marker genes were expressed in conditioned medium-treated ADSCs. *Ck19* was expressed in ADSCs cultured in adult, E13.5, and E19.5 mouse hepatocyte derived conditioned medium (Fig. 3). *Ck7* was expressed in ADSCs cultured in adult, neonate, E13.5, E15.5 and E19.5 mouse hepatocyte-derived conditioned medium (Fig. 3). Furthermore, *Sox9*, which was expressed in various types of

stem cells or progenitor cells, was expressed in undifferentiated ADSCs and conditioned medium-treated ADSCs (Fig. 3). These data suggested that the conditioned culture medium from mouse fetal hepatocyte could induce mouse ADSCs to differentiate to bile cell lineages and/or their progenitor cells.

Discussion

The present study showed that conditioned medium derived from hepatocytes could induce ADSCs to bile cells. In mouse embryonic development, CCAAT-enhancer-binding protein α (C/EBPα) is known as a critical transcription factor that induces hepatoblasts into hepatocytes (13). Downregulation of C/EBPα is the most important event in differentiation into bile cells. C/EBPα-knockout mice could not develop mature hepatocyte cells, and all the cells that should have been hepatocytes were bile cells (14). Furthermore, it was reported that transforming growth factor β (TGFβ)/activin and Notch signaling were important for developing bile cells. Therefore, it was considered that the conditioned medium derived from hepatocytes may contain the C/EBPα inhibitor, TGFβ/activin or Notch (15-17). In conclusion, analysis of the factors in the conditioned medium will lead to the development of efficient bile cell differentiation culture medium and regenerative therapy for the bile duct.

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