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Expression of Notch signalling markers in bone marrow cells that differentiate into a liver cell lineage in a rat transplant model

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Abstract

Notch signalling pathway plays an important role in cell differentiation. To investigate the implications of Notch signalling in the differentiation of rat bone marrow (BM) cells into a liver cell lineage, we used cultured BM cells to examine the mRNA expression of Musashi-1, which positively regulates Notch signalling, and made a transplant model to examine the protein expression of Notch signalling markers. For the in vivo experiment, BM cells were collected from transgenic rats expressing green fluorescence protein (GFP) and transplanted into the spleens of recipient rats, in which liver damage had been induced with carbon tetrachloride. The expression of Notch receptor 1 (Notch-1), Jagged-1 and Musashi-1, in the transplanted GFP-positive BM cells was investigated by immunohistochemistry. The expression of the liver-specific proteins, alpha-fetoprotein and cytokeratin19 was also investigated. Musashi-1 mRNA became detectable in the BM cells on culture day 7 in vitro. After transplantation, GFP-positive BM cells were observed in the portal areas of the recipient's livers. Notch-1, Jagged-1, Musashi-1, alpha-fetoprotein and cytokeratin19 were all expressed in the transplanted BM cells. These results suggest that the Notch signalling pathway plays a role in the differentiation of BM cells into a liver cell lineage.

Keywords: Bone marrow; Differentiation; Notch-1; Jagged-1; Musashi-1; Transplantation; Carbon tetrachloride

1. Introduction

The characterization of bone marrow (BM) cells is of great scientific interest in the field of liver regenerative medicine [1–3]. In vitro studies have shown that BM stem cells can produce albumin when cultured with hepatocyte growth factor (HGF) [4], while those with the β2m-/Thy-1+ phenotype can produce both albumin and various liver-specific factors when cultured with hepatocytes [5]. Further in vivo studies have shown that a population of rat BM stem cells can differentiate into hepatic cells [6]. These findings indicate that BM cells have a potential role in liver regeneration.

We previously devised a negative selective magnetic cell separation system for sorting rat BM cells containing a hematopoietic stem cell-enriched fraction [7]. We then demonstrated that these stem cell-enriched BM cells have the potential to differentiate into a liver cell lineage, and found some evidence to suggest that Notch signalling plays a role in the early stages of such differentiation in vitro [7]. In the present study, we further investigated the expression of mRNA for Notch receptor-1 (Notch-1) and its ligands Jagged-1 and Musashi-1 in cultured BM cells destined to differentiate into cells expressing liver-specific genes.

To demonstrate the differentiation of BM cells into cells expressing liver-specific proteins in the liver in vivo, stem cell-enriched BM cells isolated from transgenic rats expressing green fluorescence protein (GFP-Tg rats) [8] were transplanted into the spleens of recipient rats, in which liver injury

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had been induced with carbon tetrachloride (CCl_4) [9,10]. The transplanted BM cells were subsequently detected in the livers of the recipients, and expressed liver-specific proteins. We investigated the in vivo expression of Notch-1, Jagged-1 and Musashi-1 in these cells using immunohistochemical methods, and showed that these Notch signalling markers were expressed in the BM cells that had differentiated to express liver-specific proteins in vivo. These findings provide further evidence of the association of Notch signalling with BM cell differentiation into a liver cell lineage.

2. Materials and methods

2.1. BM cell culture

The preparation and purification of the hematopoietic stem cell-enriched BM cells was performed according to our previously described method [7]. Briefly, BM cells were obtained by flushing out the femurs of 8-week-old male Sprague-Dawley (SD) rats. A negative selective magnetic cell separation system was used to separate out the hematopoietic stem cell-enriched BM cells, which were then suspended in phosphate-buffered saline, pH 7.2 (PBS). Normal adult hepatocytes were isolated by the perfusion method of Seglen [11] with some modifications [12]. The viability of the cells was always greater than 85%, as judged by trypan blue exclusion. For co-culture of the sorted BM cells with the hepatocytes, we used 6-well culture plates in which the two chambers were separated by a semi-permeable membrane (Transwell, Corning Coaster Corporation, Cambridge, MA; pore size 0.4 μm). Hepatocytes ($3.0 \times 10^5/cm^2$) and sorted BM cells ($1 \times 10^5/cm^2$) were plated in the upper and lower chambers, respectively. The cells were cultured with Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 10^{-7} M dexamethasone, 0.5 μg/ml insulin and 100 U/ml penicillin. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. RNA isolation

The BM cells that had been cultured with the hepatocytes were harvested on culture days 0, 3 and 7. Their total cellular RNAs were extracted using an Isogen kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instructions. The RNA concentrations were determined by ultraviolet spectrophotometry (UV-1200 spectrophotometer, Shimazu, Kyoto, Japan).

2.3. Reverse transcription-polymerase chain reaction

The expression of mRNAs for the Notch signalling markers was examined on culture days 0, 3 and 7 using the reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was carried out using the superscript first-strand synthesis system (Invitrogen, Carlsbad, CA). PCR was performed over 35 cycles at a denaturation temperature of 94 °C for 1 min, an annealing temperature of 58 °C for 1 min, and an extension temperature of 72 °C for 1 min using a Perkin-Elmer 9600 thermal cycler platform (Perkin-Elmer, Norwalk, CT). The primers used to detect the mRNAs for the various Notch signalling markers were as follows: Notch-1 gene: 5′-GACTATGCCTGCAGCTGTGCC-3′ and 5′-GGCTGCAGGGCACGTAGG-3′ (PCR product; 421 bp); Jagged-1 gene: 5′-CTACATAGCCTGTGAGCCTTC-3′ and 5′-ATATCATCCTCTTCCACTTCC-3′ (PCR product; 492 bp); Musashi-1 gene: 5′-GTACCCATTGGTGAAGGCTGTGGCA-3′ and 5′-CAAGATGTTCATCGGGGGACTCAGTT-3′ (PCR product; 1024 bp). Additional nested primers for PCR of the Musashi-1 gene were 5′-GGCTTCGTCACTTTCATGGACCAGGCG-3′ and 5′-GGGAACTGGTAGGTGTAAC-3′ (PCR product; 542 bp). The GAPDH gene was used as a control for the PCR; the GAPDH primers were 5′-ATCACTGCCACTCAGAAGAC-3′ and 5′-TGAGGGAGATGCTCAGTGTT-3′ (PCR product; 580 bp).

2.4. BM cell transplantation experiment

The animal study protocol was approved by the Animal Studies Ethics Committee of Yamagata University School of Medicine, Japan. The BM cell donors were 8-week-old male GFP-Tg rats (8-week-old), provided by Japan SLC, Inc. (Hamamatsu, Japan) and used with the kind agreement of Prof. Dr. Masaru Okabe (Genome Information research Center, Osaka University, Japan). The BM cell recipients were 8-week-old male SD rats obtained from Japan SLC Inc.; they were housed in a temperature-monitored environment (20–22 °C) with a 12 h light–dark cycle, and fed standard rat chow ad libitum. The three recipient rats received 0.8 ml/kg CCl_4 diluted 1:1 with corn oil and administered by gavage, twice a week for 2 weeks before transplantation.

The BM cells for transplantation were isolated from the GFP-Tg rats, and hematopoietic stem cell-enriched, purified BM cells were obtained using our previously described negative selective magnetic cell separation system [7]. Each recipient received 5×10^6 cells, which were transplanted into the spleen with a syringe needle according to the procedure reported by Kobayashi et al. [13]. A 20% partial hepatectomy was performed immediately after BM cell transplantation. As an experimental control, 1 ml PBS instead of BM cells was transplanted into the spleens of two rats that had been treated with CCl_4. The recipient rats were subsequently fed CCl_4 (0.8 ml/kg) twice a week for 2 weeks, and were sacrificed 14 days after the final CCl_4 administration.

2.5. Immunohistochemistry

In order to identify the transplanted BM cells in the recipients' livers, they were first observed under a fluorescence microscope (Olympus BX-FLA; Olympus Optical, Tokyo, Japan), then immunostained using antibodies specific to GFP.