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Biochemical and Biophysical Research Communications 298 (2002) 24–30
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Improved conditions to induce hepatocytes from rat bone marrow cells in culture

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Received 4 September 2002

Abstract

Recent studies have revealed that bone marrow cells can develop into hepatocytes by in vivo transplantation under certain circumstances. However, little is known about the mechanism of bone marrow cell differentiation into hepatocytes. It is important to determine suitable culture conditions in which bone marrow cells will be differentiated into hepatocytes not only for understanding differentiation mechanisms but also for efficient amplification of hepatocyte-progenitor cells of bone marrow origin, this being a prerequisite for potential therapeutic use. In the present study, we found that hepatocyte growth factor (HGF) receptor (c-Met)- and α-fetoprotein-expressing cells were present in adult rat bone marrow. We also found that these cells also express hematopoietic stem cell markers, such as CD34, Thy-1, and c-Kit. Using an HGM medium with HGF and EGF, we succeeded in propagating hepatocyte-like cells induced from adult rat bone marrow in culture. These cells were immunocytochemically stained for albumin. By RT-PCR analysis of cultures containing the hepatocyte-like cells, we detected mRNAs of tryptophan-2,3-dioxygenase and tyrosine aminotransferase, markers of hepatocytes at a terminal differentiation stage. The present culture therefore can be a useful resource for cell transplantation therapy for liver diseases.

Keywords: Bone marrow cells; Hepatocytes; Albumin; Tryptophan-2,3-dioxygenase; Tyrosine aminotransferase

Recently accumulating evidence indicates that bone marrow includes a large variety of progenitor cells that can differentiate into specific cell types. Transplantation of bone marrow cells in vivo resulted in the appearance of differentiated cells of donor origin in various recipient tissues, including skeletal myoblasts [1,2], cardiac myoblasts [3,4], endothelium [4,5], lung, gut, and skin epithelia [6], and neuroectodermal cells [7].

Transplanted bone marrow cells can also repopulate hepatic and biliary duct epithelia under certain circumstances [8–11]. When bone marrow cells from male rats were transplanted into lethally irradiated syngeneic female rats whose liver was severely injured afterwards with 2-acetylaminofluorene and CCl_4, donor-derived hepatocytes, biliary cells, and/or oval cells were detected in the recipient liver [8]. Similar results have been reported for mice and human patients [9,10]. Furthermore, transplantation of bone marrow cells into fumarylacetoacetate hydrolase (FAH)-deficient mice, an animal model of human fatal congenital tyrosinemia type I, rescued the mice and restored their liver functions [11]. Thus, bone marrow cells have a therapeutic potential for acute and chronic liver failure. For practical therapeutic use, however, it is necessary to prepare an enormous amount of bone marrow-derived hepatocytes with highly differentiated functions. In this context, it is important to establish a culture method that allows bone marrow cells to actively grow and fully differentiate into hepatocytes.

Cell growth and differentiation are mainly controlled by growth factors and cytokines. In order to establish a culture method for induction of differentiation of bone

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PII: S0006-291X(02)02340-9

marrow cells into hepatocytes, we focused on hepatocyte growth factor (HGF). HGF was originally identified and cloned as a potent mitogen for hepatocytes [12,13], and it has been shown to play essential roles in the development and regeneration of the liver [14,15]. HGF has been shown to have mitogenic, motogenic, and morphogenic activities toward a large variety of cells other than hepatocytes that express its receptor c-Met [16]. In a previous study, we found that bone marrow cells of adult rats include c-Met-expressing cells and that the in vitro treatment of bone marrow cells with HGF induces albumin-expressing hepatocyte-like cells from them. These cells were found to express cytokeratins 8 and 18, which are typically expressed in normal adult hepatocytes [17]. In the present study, we improved culture conditions to enable efficient propagation of bone marrow-derived hepatocytes and expression of tryptophan-2,3-dioxygenase (TO) and tyrosine aminotransferase (TAT), markers of hepatocytes at a terminal differentiation stage, in addition to albumin, a marker of hepatocytes at an earlier differentiation stage.

Materials and methods

Cell culture. Bone marrow cells were collected from the femora of Wistar rats (7–9 weeks old). The marrow cells were inoculated at a density of $8 \times 10^5/cm^2$ into 100-mm plastic dishes (for RNA extraction) or 24-well plates with round glass coverslips of 14 mm in diameter (for immunocytochemical staining). These plastic dishes and glass coverslips were pre-coated with 0.3% type I collagen extracted from the rat tail tendon, as described previously [17]. Basal media used were DF medium [a mixture (1:1) of Dulbecco's modified Eagle's medium (DMEM 05919, Nissui Paharmaceutical, Tokyo, Japan) and Ham's medium F12 (Nissui)], and HGM medium, a medium that was developed to grow primary hepatocytes [18,19]. The HGM medium was slightly modified in the present study, i.e., DMEM supplemented with bovine serum albumin 2.0 g/L, glucose 1.0 g/L (finally 2.0 g/L), galactose 2.0 g/L, ornithine 0.1 g/L, proline 0.030 g/L, glutamine 0.730 g/L (finally 5.0 mM), nicotinamide 0.610 g/L, $ZnCl_2$ 0.544 mg/L, $ZnSO_4 \cdot 7H_2O$ 0.750 mg/L, $CuSO_4 \cdot 5H_2O$ 0.20 mg/L, $MnCl_2 \cdot 4H_2O$ 0.0327 mg/L, ITS 10 mL/L (Gibco BRL, Gaithersburg, MD, insulin 1.0 g/L, sodium selenite 0.67 mg/L, transferrin 0.55 g/L, and sodium pyruvate 11.0 g/L), dexamethasone 0.1 μM, and Hepes 10 mM (pH 7.2). The HGF used was the Δ5 variant [20], a mature two-chain form, kindly provided by Snow Brand Milk Products Co., Ltd. (Tokyo). EGF was purchased from Sigma. HGF and EGF were freshly added when the medium was changed every 3 days.

Hepatocytes were isolated from Wistar rats by a conventional two-step collagenase liver perfusion [19] and cultured in collagen-coated plastic dishes containing DMEM supplemented with 1 μM dexamethasone, 1 μM insulin, and 10% FBS.

Rat skin fibroblasts prepared from dermal tissue fragments were cultured in DMEM supplemented with 10% FBS.

Immunocytochemical staining. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 30 min and then treated with 70% ethanol at −20 °C for 30 min. After washing with PBS, the fixed cells were incubated in a blocking solution of 10% skim milk, 6% glycine, and 0.05% sodium azide in PBS at room temperature for 30 min. The cells were then reacted at 4 °C overnight with a primary antibody, i.e., anti-mouse c-Met (Santa Cruz Biotechnology, Santa Cruz, CA), anti-human AFP (NeoMarkers, Fremont, CA), anti-human albumin (Dako, Denmark), anti-human CD34 (Santa Cruz), anti-human Thy-1 (Santa Cruz), or anti-human c-Kit (Santa Cruz) antibody, which had been diluted at 1:100 with the blocking solution. After washing with the blocking solution, the cells were incubated at 37 °C for 1 h with the second antibody, FITC- or TRITC-conjugated antibodies (Santa Cruz, 1:100 dilution with the blocking solution) and with 1 mM Hoechst 33258 (Sigma) for nuclear staining.

Detection of TO and TAT mRNAs. Total RNA was isolated from adult rat liver, cultured rat skin fibroblasts, and fresh and cultured rat bone marrow cells by the guanidinium thiocyanate-phenol method, and 1 μg RNA was used for cDNA synthesis. The resulting products were amplified under the following conditions: initial incubation at 94 °C for 4 min followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and then a final step at 72 °C for 5 min. The TO primers used were 5′-GAGCAGGAGCAGACGCTATT-3′ (sense strand) and 5′-CACCTTGTACCTGTCGCTCA-3′ (antisense strand), which delineated a 498-bp product. The nested primers 5′-AA CGCACACCTGGCTTAGAG-3′ and 5′-CTTGCTGCCTAGCAT CCTGT-3′ delineated a 389-bp product. The TAT primers used were 5′-GTCCATCGGCTACCTATCCA-3′ (sense strand) and 5′-CAGG ACAGGATGGGAACATT-3′ (antisense strand), which delineated a 492-bp product. The nested primers 5′-GGGAGGAGGTCGC TTCTTAC-3′ and 5′-GGAACATTGGTGCTGAGGTT-3′ delineated a 457-bp product. The glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, as an internal control) primers used were 5′-ATCACTGCC ACTCAGAAGAC-3′ (sense strand) and 5′-TGAGGGAGATGCTCA GTGTT-3′ (antisense strand), which delineated a 578-bp product. The amplified products were subjected to electrophoresis in 1% agarose gels and stained with ethidium bromide.

Results

By RT-PCR analysis, we previously found that bone marrow cells from adult rats included cells expressing mRNA of the HGF receptor c-Met and AFP [17]. Therefore, we first immunocytochemically examined the expression of c-Met and AFP in bone marrow cells. a small number of bone marrow cells were positively stained for c-Met protein (Fig. 1A). Interestingly, most of the c-Met-positive cells were also stained for AFP (Figs. 1A–C). Quantitatively, 3.64% and 3.92% of the bone marrow cells were positive for c-Met and AFP, respectively (Table 1). Furthermore, most of the c-Met-positive cells were also positive for CD34, Thy-1, and c-Kit (Figs. 1D–L). These results indicate that c-Met- and AFP-expressing cells may be derived from hematopoietic stem cells.

We previously reported that in vitro treatment of bone marrow cells with HGF induced albumin-expressing hepatocyte-like cells and that those cells also expressed cytokeratins 8 and 18, which are typically expressed in normal adult hepatocytes [17]. We could not detect, however, messages specific to terminally differentiated hepatocytes at more advanced stages such as TO and TAT. In addition, the fraction of hepatocyte-like cells in the bone marrow-derived cells was very small. The cells were cultivated in DF as a basal medium. Since HGM medium was developed for growing primary hepatocytes, it might also be a good medium

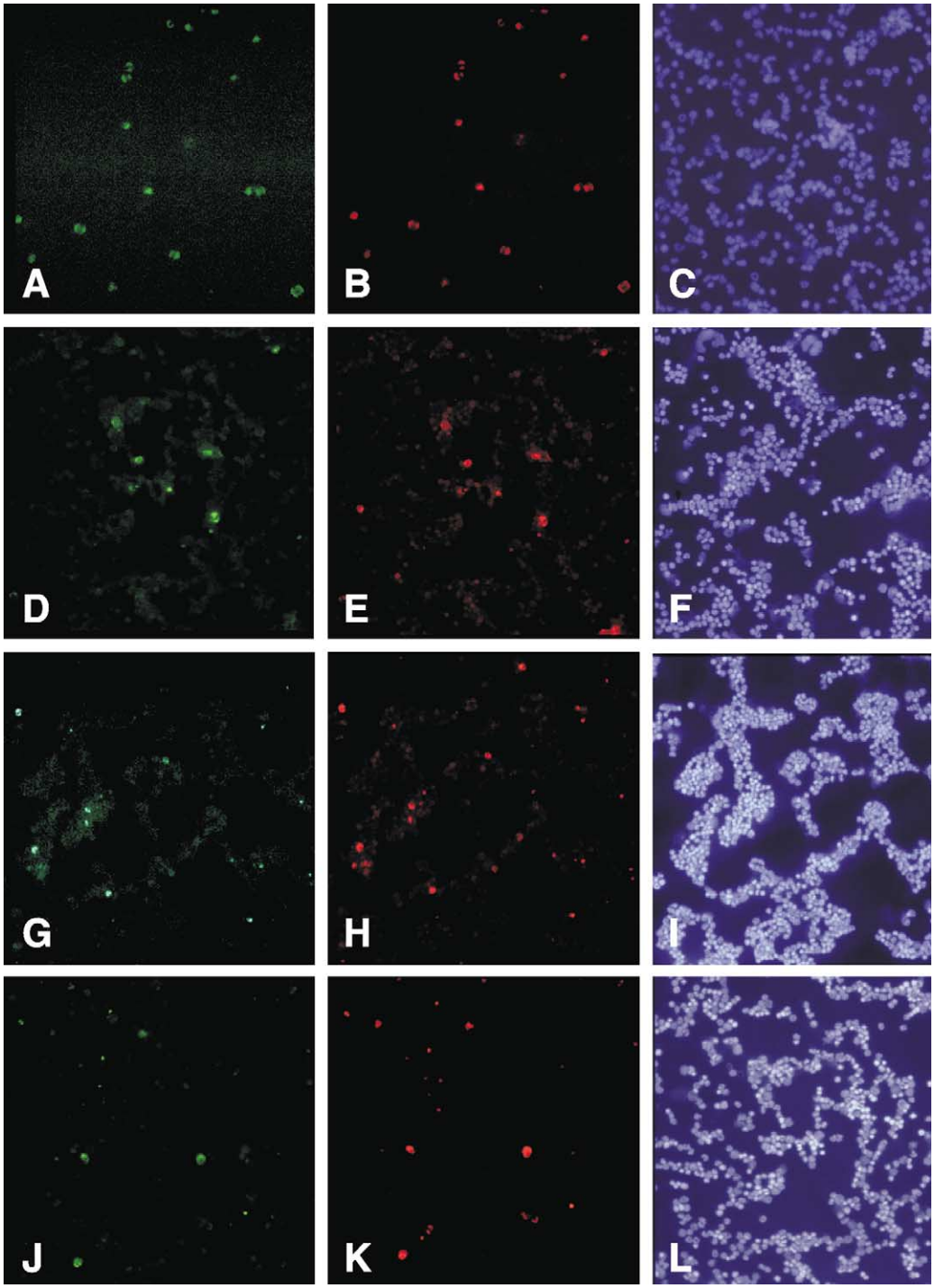

Fig. 1. Immunocytochemical staining of bone marrow cells freshly isolated from adult rats. Cells were doubly stained with anti-mouse c-Met antibody (in green: A, D, G, and J) in combination with either anti-human AFP (B), anti-human CD34 (E), anti-human Thy-1 (H), or anti-human c-Kit antibody (K) shown in red. Nuclei were stained with Hoechst 33258 (C, F, I, and L). A, B, and C; D, E, and F; G, H, and I; and J, K, and L are the same fields of smeared bone marrow cells.

for growth of cells in the process of differentiation from bone marrow cells to hepatocytes.

When bone marrow cells were cultured in HGM medium with 10% FBS in the presence of HGF (40–1000 ng/mL) and EGF (20 ng/mL) for 2 weeks, colonies composed of polygonal cells resembling mature hepatocytes were found (Figs. 2A and B). Without HGF and EGF, such colonies were still formed, but the number and sizes of colonies were far smaller than those in the presence of HGF and EGF (Figs. 2A–C). The number and sizes of polygonal cell colonies increased at higher concentrations of HGF. Morphologically, the

Table 1
Ratios of c-Met and AFP-positive cells in rat bone marrow cells

Stained protein	% Positive cells
c-Met	3.64 ± 0.75
AFP	3.92 ± 0.71

Mean ± SD from 5 rats.

polygonal cells induced from bone marrow are very similar to mature hepatocytes from adult rat in primary culture, as shown in Figs. 2E and F. The hepatocyte-like cells were positively stained for albumin (Fig. 3). Furthermore, we detected mRNA of TO and TAT by RT-PCR, markers of hepatocytes at the most advanced differentiation stage (Fig. 4).

Discussion

Medium components that promote stem cells to proliferate and differentiate into specific cell types are currently under intensive study. In the present study, we improved culture conditions to enable efficient differentiation of bone marrow cells into hepatocytes and propagation of the hepatocytes. DF medium, which we used in a previous study, contains a good balance of nutrients and has been widely used for cultivating various cell types. DF medium fairly well supported the growth and differentiation of bone marrow cells into hepatocyte-like cells in the presence of HGF [17].

HGM medium was originally developed for clonal expansion of primary hepatocytes from adult rats [18]. This medium allowed active proliferation and redifferentiation afterwards of primary hepatocytes in the presence of HGF, EGF, TGF α, and/or phenobarbital [18,19]. HGM medium contains nicotinamide, which efficiently sustains proliferation and maturation of small hepatocytes from adult rats in primary culture [21,22]. HGM medium also contains dexamethasone, which has a strong stimulatory effect on DNA synthesis of primary hepatocytes [18]. The other constituents such as ITS (insulin, transferrin, and selenium), proline, and trace elements (Zn, Mn, and Cu) have also shown to be effective in proliferation and survival of primary hepatocytes [18,23–26] and in differentiation of bone marrow cells from cholestatic rats in the presence of their serum [27]. Using HGM as a basal medium, we were able to induce the differentiation of bone marrow cells into hepatocyte-like cell colonies expressing the terminal differentiation markers TO and TAT, which were never

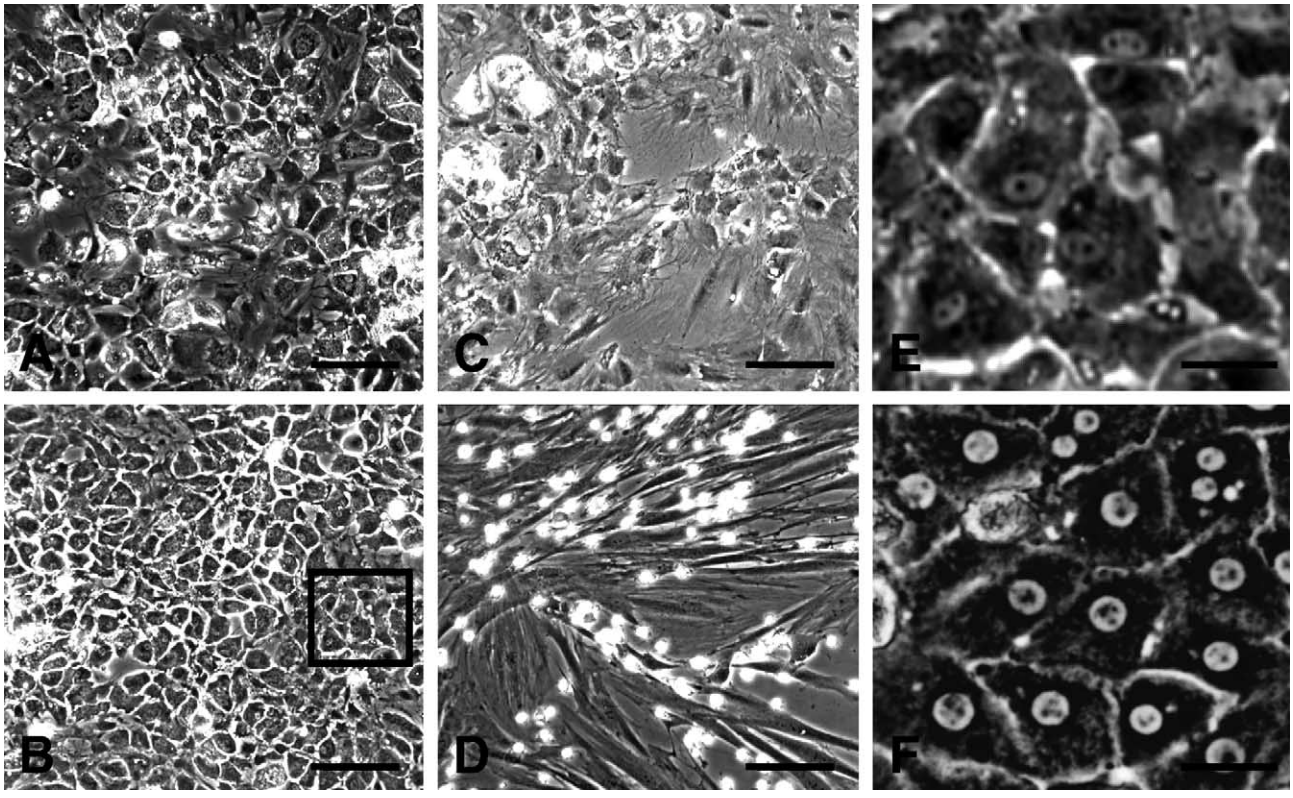

Fig. 2. Appearance of primary bone marrow cell and primary hepatocyte cultures. Bone marrow cells were cultured for 2 weeks in different media. (A) HGM + 10% FBS + EGF 20 ng/ml + HGF 40 ng/ml; (B) HGM + 10% FBS + EGF 20 ng/ml + HGF 1000 ng/ml; (C) HGM + 10% FBS; (D) DMEM + 10% FBS; (E) shows the same picture as that in (B) at a higher magnification; and (F) primary adult rat hepatocytes on culture day 1. Bars indicate 100 μm (A, B, C, D) or 25 μm (E, F).

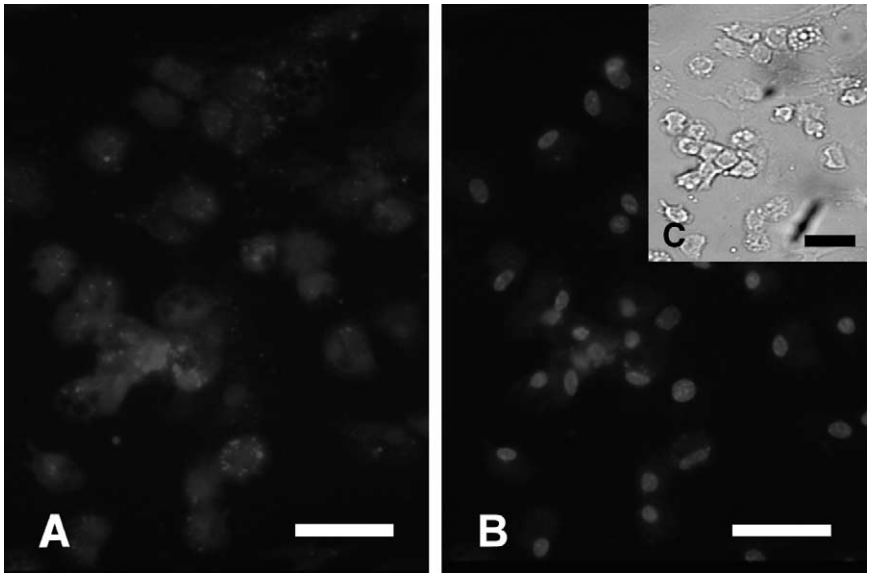

Fig. 3. Immunocytochemical staining of albumin in hepatocyte-like cells induced from bone marrow cells cultured for 2 weeks in HGM medium supplemented with HGF (1000 ng/ml), EGF (20 ng/ml), and 10% FBS. (A) Stained with anti-human albumin; (B) stained with Hoechst 33258; and (C) light microscopic image. A, B, and C show the same field of the culture. Bars indicate 50 μm.

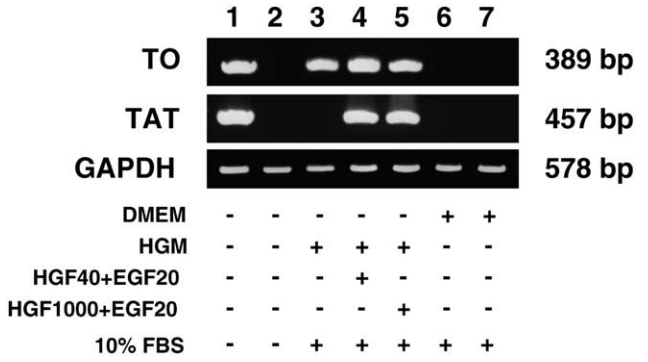

Fig. 4. Expression of TO and TAT in bone marrow cell cultures under various conditions. Total RNA samples were prepared from adult rat liver (lane 1, as a positive control); fresh bone marrow cells (lane 2); 2-week cultures of bone marrow cells in HGM medium (lanes 3–5) or DMEM supplemented with 10% FBS (lane 6) in the presence (lanes 4 and 5) or absence (lanes 3 and 6) of HGF [40 (lane 4) or 1,000 ng/ml (lane 5)] and EGF [20 ng/ml (lanes 4 and 5)]; and rat skin fibroblasts (lane 7, as a negative control). These samples were analyzed by RT-PCR for detection of TO, TAT, and GAPDH mRNAs.

detected in the cultures using DF medium. In addition, the number and sizes of those colonies were far larger in HGM than those in DF medium. These results indicate that HGM is more suitable for inducing hepatocyte-like cells from bone marrow.

Characterization of hepatic progenitor cells in bone marrow is of great scientific and clinical interest. Lagasse et al. have shown that hematopoietc stem cells exclusively contribute to the production of hepatocytes in the regenerating liver of FAH-deficient mice after bone marrow cell transplantation [11]. In the present study, we did not identify the progenitor of hepatocyte-like cells. However, it is reasonable to assume that the induced hepatocyte-like cells were derived from c-Met-positive cells since HGF was essential for the induction and c-Met is the only known receptor of HGF. We found that most of the c-Met- and AFP-positive cells also expressed hematopoietic stem cell markers CD-34, Thy-1, and c-Kit (Figs. 1D–L). Taken together, the results suggest that the hepatocyte-like cells induced in the present culture system originate from hematopoietic stem cells. Purification of c-Met- and AFP-expressing cells from bone marrow, which is now under the way, should provide a clearer answer.

It has very recently been reported that mesenchymal stem cells from adult murine marrow also differentiated into hepatocyte-like cells in culture in the presence of fibroblast growth factor (FGF)-4 and HGF as well as after in vivo transplantation [28,29]. The phenotype of mesenchymal stem cells is different from that of hematopoietic stem cells and the former cells express a low level of Thy-1 but not CD34 and c-Kit [28]. Thus, there may be at least two stem cell lineages that contribute to the production of hepatocytes in vivo and in vitro. At present, their relative contribution upon transplantation of unfractionated bone marrow cells is not clear.

For practical therapeutic use, a method by which an enormous amount of differentiated hepatocytes, preferably of autologous origin, can be produced is needed. Bone marrow-derived hepatocytes have great potential utility since the therapeutic target patients are likely to be suffering from liver failure and to have difficulty in producing healthy hepatocytes. The present study is a step further in such a direction, but we were still only able to propagate a small number of hepatocyte-like

cells from bone marrow. Many growth factors and cytokines, most notably HGF, EGF, transforming growth factor-α, interleukin-6, tumor necrosis factor-α, insulin, and norepinephrine, appear to play important roles in liver regeneration [15]. FGFs, oncostatin M, and several families of transcription factors, including hepatocyte nuclear factors 1, 3, and 4, have been shown to be important components of liver development and differentiation processes [30]. Furthermore, heterotypic cell interaction modulates cell growth, migration, and/or differentiation. In both the developing and adult liver, cell–cell interactions are imperative for coordinated organ function [31]. Thus, it is important to further improve culture conditions for production of a large number of bone marrow-derived hepatocytes by an appropriate combination of growth factors, cytokines, and cell–cell interactions.

In conclusion, c-Met- and AFP-positive cells are present in adult rat bone marrow. These cells also express hematopoietic stem cell markers, such as CD34, Thy-1, and c-Kit. It is possible to induce the production of cells that express terminally differentiated hepatocyte markers from bone marrow using an HGM medium containing HGF and EGF. These cultures may be useful resources for cell transplantation therapy for patients with liver diseases.

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