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Hepatic differentiation induced by oncostatin M attenuates fetal liver hematopoiesis

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ABSTRACT **Embryonic liver is a transient site for definitive hematopoiesis. Along with maturation of the bone marrow and spleen, hematopoietic cells relocate from the liver to their final destinations while the liver starts organizing its own structure and develops numerous metabolic functions toward adult. Recently, it was demonstrated that the signal exerted by oncostatin M (OSM) through gp130 plays a pivotal role in the maturation process of the liver both *in vitro* and *in vivo*. However, the molecular basis underlying the termination of embryonic hematopoiesis remains unknown. In this study, we report that primary culture of fetal hepatic cells from embryonic day 14.5 murine embryos supported expansion of blood cells from $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ cells, giving rise to myeloid, lymphoid, and erythroid lineages. Of interest, promotion of hepatic development by OSM and glucocorticoid strongly suppressed *in vitro* hematopoiesis. Consistent with these results, hepatic culture from the embryonic day 18.5 liver no longer supported hematopoiesis. These data together with the previous observations suggest that the signals exerted by OSM and glucocorticoid induce hepatic differentiation, which in turn terminate embryonic hematopoiesis and promote relocation of hematopoietic cells.**

Adult liver performs various metabolic functions: serum protein synthesis, lipogenesis, detoxification, and urea synthesis among many others. In contrast, the embryonic liver has much less metabolic activity and, rather, functions as a hematopoietic microenvironment (1–4). Increasing evidence indicate that hematopoietic stem cells (HSCs) originating from the aorta-gonad-mesonephros (AGM) region migrate to the fetal liver to generate numerous definitive hematopoietic cells. In the murine system, the initial definitive hematopoietic cells were identified in the AGM region as early as embryonic day 10.5 (E10.5) (5–7) or even earlier in the para-aorta splanchnopleura (8) or yolk sac (9). Subsequently, the fetal liver becomes the major site for hematopoiesis from E12 through E16. Although the majority of liver metabolic functions appears peri- or postnataly, hematopoiesis-supporting activity of the liver is lost during late-fetal development. Finally, hematopoietic cells move into the bone marrow or spleen around the perinatal stage to constitute the adult-type hematopoietic system. So far, the molecular basis underlying the switching of liver functions around the perinatal stage remains unexplored.

The establishment of a novel primary culture of fetal hepatic cells derived from E14.5-murine embryos that retains many characteristics of fetal liver cells *in vivo* was recently reported (10). Using this system, it was demonstrated that murine oncostatin M (OSM) (11) stimulated progression of hepatocytic development, as evidenced by the morphology and by induction of marker genes for the postnatal liver such as glucose-6-phosphatase and tyrosine amino transferase. Furthermore, OSM stimulated glycogenesis, lipogenesis, and clearance of ammonia from culture media, indicative of functional maturation of hepatic parenchymal cells (N. Kojima, T. Kinoshita, A. Kamiya, and A. Miyajima, unpublished work). In agreement with these observations, knockout mice deficient for gp130 (12), a signaling component of the OSM receptor (13–15), display defective maturation of the liver (10). Of interest, OSM is expressed in $CD45^{+}$-hematopoietic cells in the embryonic liver whereas adherent hepatocytes predominantly express the OSM-specific receptor. These results strongly suggested that OSM is a paracrine mediator playing a pivotal role in hepatic development from late-fetal to neonatal stage.

In this paper, we took advantage of our primary culture system of fetal hepatic cells and attempted to reconstitute embryonic hematopoiesis *in vitro*. We found that fetal hepatic cells in primary culture support expansion of blood cells from HSCs in the presence of stem cell factor (SCF), generating multiple lineages of hematopoietic cells, including myeloid, erythroid, and lymphoid cells. Surprisingly, the activity of stromal hepatic cells to support *in vitro* hematopoiesis, particularly erythropoiesis, was strongly suppressed along with the progression of hepatic development induced by OSM. Our results indicate that OSM is capable of not only stimulating maturation of hepatic parenchymal cells but also terminating the embryonic liver function as a hematopoietic microenvironment.

MATERIALS AND METHODS

Materials. C57BL/6CrSlc mice (Nihon SLC, Hamamatsu, Japan) were used in all experiments. FCS, liver perfusion medium, liver digest medium, nonessential amino acid solution, and insulin were from GIBCO/BRL. Antibodies to label hematopoietic cells were purchased from PharMingen, and murine OSM was from R & D Systems.

Cell Culture. Primary culture of adherent hepatic cells (a stromal layer) was prepared as follows. The liver tissues derived from E14.5-murine embryos were minced into pieces (<1 mm^3) and were incubated with perfusion medium containing EGTA followed by enzyme-based dissociation medium (liver digest medium) with gentle agitation. Tissue pieces were gently pipetted, were subjected to hemolysis with hypotonic

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Abbreviations: OSM, oncostatin M; Dex, dexamethasone; HSC, hematopoietic stem cell; AGM, aorta-gonad-mesonephros; E10.5, embryonic day 10.5; SCF, stem cell factor; cfu, colony-forming unit; Epo, erythropoietin; M-CSF, macrophage colony-stimulating factor.
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buffer, and were filtered through a cell strainer (Falcon) to remove cell debris. Hepatic cells were suspended in the culture medium composed of DMEM supplemented with 10% FCS, 2 mM L-glutamine, 1× nonessential amino acid solution, 50 μM 2-mercaptoethanol, and antibiotics and were inoculated onto 0.1% gelatin-coated Primaria cell-culture dishes (60-mm diameter, Falcon) at 4×10^5 cells/dish cell density. In the standard culture condition, 1×10^{-7} M dexamethasone was added to support hepatic cells unless indicated. Cells were cultured at 37°C in a CO_2 incubator (20% O_2/5% CO_2) for several hours, and floating or dead cells were removed by extensive washing with PBS or culture medium. HSCs (Lin^-[Gr-1^-/B220$^-$/CD4$^-$/CD8$^-$/TER119$^-$]Sca-1^+c-Kit^+ cells) (16) derived from E14.5-murine embryos were isolated by a two-step protocol: Dynabeads-based negative selection (Dynal, Oslo) followed by positive selection using the FACS-Vantage system (Beckton Dickinson). One-thousand Lin^-Sca-1^+c-Kit^+ hematopoietic cells were inoculated onto a subconfluent monolayer of primary fetal hepatic cells grown in 60-mm diameter dishes. After 2 days of incubation, floating or dead cells were removed by washing. Cells then were stimulated with SCF and were incubated for 8 days.

Colony-Forming Unit (cfu) Assays. Hematopoietic progenitors were analyzed for their potential to form colonies in methylcellulose-containing media either in the presence of IL-3, IL-6, erythropoietin (Epo), and SCF for myeloid lineages, Epo alone for erythroid lineage, or IL-7 and SCF for the B-lymphoid lineage.

Analysis of Cell Surface Markers. Floating cells generated on fetal hepatic culture were recovered and stained with antibodies specific for hematopoietic cell surface markers. Stained cells then were analyzed by the FACS-Calibur system (Beckton Dickinson).

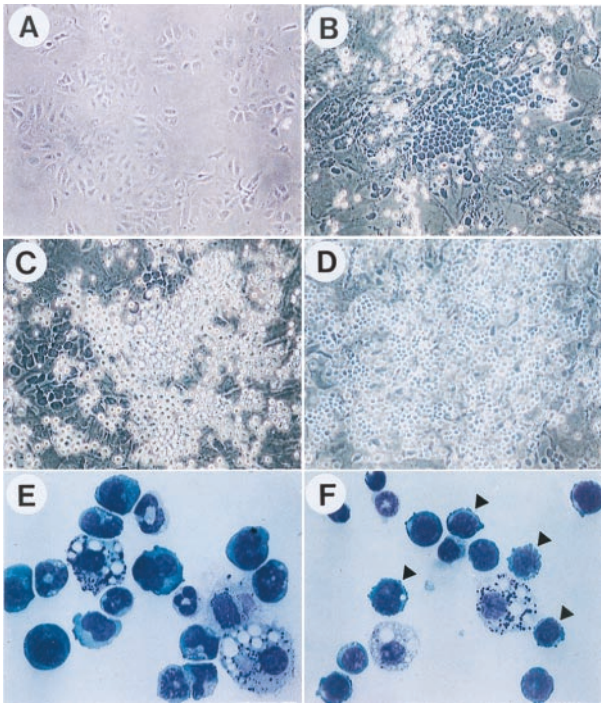

FIG. 1. Characterization of hematopoietic cells generated in the co-culture. Fetal liver-derived HSCs (E14.5) were cultured on primary fetal hepatic cells for 10 days in the presence (*B*, *C*, and *E*) or absence of Dex (*D* and *F*). (*A*) Morphology of the fetal hepatic culture (E14.5) just before the addition of HSCs. (*B*) Typical cobblestone area found in the co-culture. (*C* and *D*) Two different floating cell types found in the co-culture. (*E* and *F*) Cytospin preparations of cells from the Dex-plus (*E*) or Dex-free (*F*) culture. Arrowheads indicate the cells specifically appeared in the Dex-free culture.

Total RNA Extraction and Northern Blot Analysis. Total RNA samples from cultured cells were extracted by the acid guanidinium thiocyanate/phenol/chloroform method (17). Expression of mRNA for hematopoietic factors was examined by Northern blot analysis. Complementary DNA probes for mouse macrophage colony-stimulating factor (M-CSF) were a kind gift from T. Sudo (Toray Industry, Kamakura, Japan) and for macrophage chemoattractant protein 1 were cloned by reverse transcription–PCR based on the reported sequence (31).

Assay for the Chemotactic Activity. Chemotactic activity in conditioned media was measured by an *in vitro* two-chamber migration assay according to a previous publication (18). In brief, 100 μl of cells in the standard culture medium were added to the upper chamber of Costar Transwells (6.5-mm diameter, 5-μm pore size, polycarbonate membrane), and hepatic conditioned media (±OSM) were added to the lower chamber to form concentration gradients. A total of 5×10^5 bone marrow-derived cells were added to the upper chamber of the Transwell and were incubated 4 h for total hematopoietic cell migration. Cells that had migrated to the lower chamber were collected and counted by FACS-Calibur (Beckton Dickinson).

RESULTS

Generation of Multilineages of Hematopoietic Cells on Primary Fetal Hepatic Cell Culture. A subconfluent monolayer of fetal liver cells from E14.5 embryos (Fig. 1*A*) was overlaid with immature hematopoietic cells (Lin^-Sca-1^+c-Kit^+ hematopoietic cells derived from the E14.5-embryonic liver) as a source of blood cells. Exogenously added Lin^-Sca-1^+c-Kit^+ hematopoietic cells adhered to or moved underneath the hepatic monolayer and formed multiple cobblestone areas, which are characteristics of immature blood cells (Fig. 1*B*). Moreover, administration of SCF resulted in production of numerous floating cells in addition to cobblestones (Fig. 1

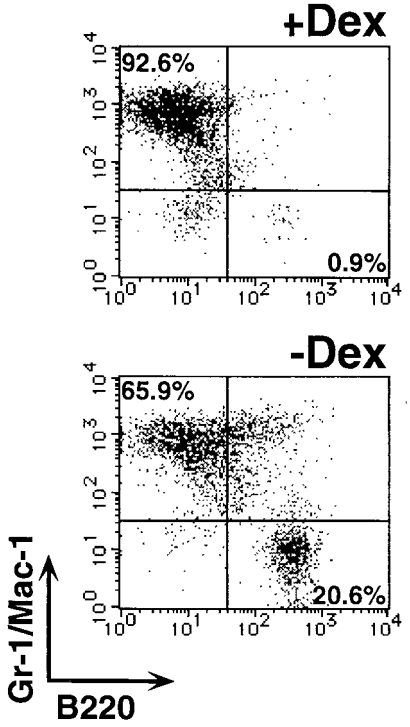

FIG. 2. Expression of surface markers on cells generated in the Dex-plus (*Upper*) or Dex-free culture (*Lower*). Vertical axis represents a mixture of myeloid lineage markers, Gr-1 and Mac-1, and horizontal axis shows a lymphoid lineage marker, B220.

Table 1. Colony-forming activities of cells generated in the co-culture system

Culture conditions	Colony types, colony number/10^4 cells					
	G	M	GM	Mix	Bfu-e	cfu-B
SCF	12.3 ± 1.0	34.7 ± 4.0	3.0 ± 1.0	0.3 ± 0.6	0.3 ± 0.6	150.7 ± 3.5
SCF + OSM	14.7 ± 2.5	50.3 ± 5.5	6.3 ± 2.1	2.0 ± 0.0	1.0 ± 1.0	n.t.
SCF + Dex	46.3 ± 6.7	26.3 ± 3.5	22.3 ± 3.1	6.7 ± 1.6	9.3 ± 0.6	10.0 ± 1.7
SCF + Dex + OSM	36.7 ± 3.2	24.0 ± 2.0	23.3 ± 2.5	11.7 ± 3.1	8.0 ± 2.0	n.t.

Shown are cfu-c and cfu-B activities of hematopoietic cells generated on the fetal hepatic culture. Floating hematopoietic cells generated in culture conditions as indicated were tested for their potential to form colonies in methylcellulose-containing media in the presence of SCF, IL-3, IL-6 and Epo for cfu-cs, or SCF and IL-7 for cfu-B. On day 10, numbers of each colony type were counted by microscopic observation. Data represent the mean values of at least triplicate experiments ± SD. n.t., not tested; G, granulocyte; M, monocyte/macrophage; Bfu-e, burst-forming unit erythroid.

C–F). These floating cells expressed cell-surface markers of hematopoietic cells such as Gr-1 and/or Mac-1 (Fig. 2 *Upper*) and contained relatively immature cells, as demonstrated by the ability to form cfu-mix in methylcellulose colony assays (Table 1). When cells were cultured in the absence of glucocorticoid [dexamethasone (Dex)], a distinct cell population of small, uniform-sized, and dark cytoplasm appeared (Fig. 1 *D* and *F*). These cells were morphologically lymphocytic (Fig. 1*F*, arrowheads), expressed a lymphoid specific cell-surface marker, B220 (Fig. 2 *Lower*), and contained immature cells capable of forming B-cell colony (cfu-B) in the presence of IL-7 and SCF (Table 1). These results indicate the presence of the lymphoid cell lineage in the Dex-free culture. The colony-forming profile of each culture (i.e., with Dex or without Dex) suggested that the Dex-plus culture tends to produce myeloid cells whereas the Dex-free culture is more lymphophilic. Although the erythroid lineage was rarely seen in either standard (plus Dex) or Dex-free culture condition, addition of Epo allowed generation of TER119-positive erythroid cells (see Fig. 3*C*). In contrast, such expansion of hematopoietic cells was not observed in stroma-free culture even if a sufficient amount of SCF was provided (data not shown). We also noted that adult bone marrow-derived HSCs could be expanded in this co-culture system (data not shown). Thus, our primary culture system reconstitutes fetal liver hematopoiesis, capable of generating myeloid, lymphoid, and erythroid lineages from HSCs.

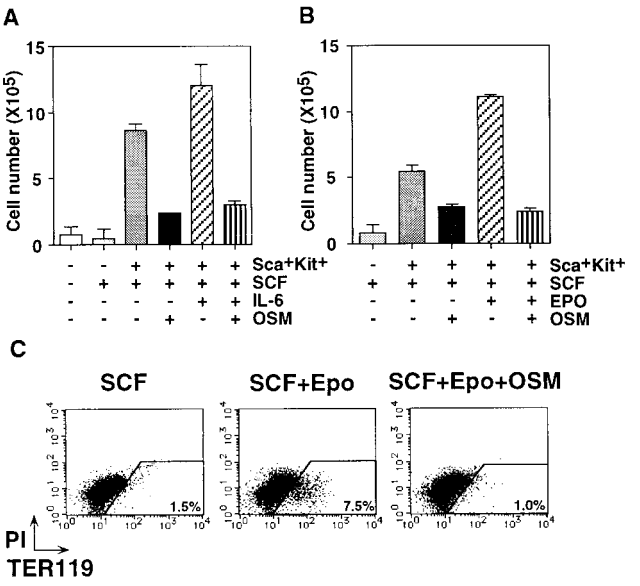

FIG. 3. Expansion of hematopoietic cells on the fetal hepatic culture and its inhibition by OSM. One-thousand $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ cells were incubated on fetal hepatic culture in the presence of SCF with or without either IL-6 (*A*) or Epo (*B*) for 10 days, and the floating hematopoietic cells were counted. Note that the presence of OSM strongly suppressed expansion of hematopoietic cells induced either by SCF alone or a combination of SCF/IL-6 or SCF/Epo. Each histogram represents the mean value of triplicate experiments ± SD of the absolute number of cells generated in 60-mm-diameter dishes. (*C*) Percentages of TER119-positive cells generated in the presence of SCF alone (*Left*), SCF/Epo (*Center*), or SCF/Epo/OSM (*Right*). PI; Propidium iodide used to eliminate fluorescence derived from dead cells.

Suppression of *in Vitro* Hematopoiesis by OSM-Induced Hepatic Development. We next examined whether *in vitro* hematopoiesis was affected by progression of hepatic development. We added mouse OSM (11) into the co-culture system to induce hepatic development and counted the number of floating cells after a 10-day incubation. In the absence of OSM, 6 to $\approx 8 \times 10^5$ floating cells were generated from 10^3 input $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ cells, and no such expansion was observed in the culture without added $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ cells. IL-6 and Epo, growth stimulators of hematopoietic cells, further increased the number of hematopoietic cells produced. In contrast, OSM drastically suppressed the expansion of hematopoietic cells, and only a few floating cells were observed (Fig. 3 *A* and *B*). Moreover, OSM inhibited enhancement of blood cell expansion by IL-6 and Epo. Notably, OSM suppressed not only the total number of cells produced in the presence of Epo but also the percentage of TER119-positive erythroid population (Fig. 3*C*). Consistently, the number of erythroid progenitors (cfu-e) produced in the presence of OSM was greatly reduced (Table 2). This inhibitory effect was not caused by a direct action of OSM on erythroid cells because addition of OSM during colony assays did not affect the number of cfu-e. It is therefore likely that hematopoiesis-supporting activity of the liver is required particularly for generation of the erythroid lineage in response to Epo. On the other hand, frequencies of myeloid progenitors were not greatly altered by the addition of

Table 2. Erythroid colony-forming activities

Culture conditions	cfu-e/10^4 cells	cfu-e/dish
SCF	3.0 ± 0.8	534 ± 138
SCF/OSM	3.3 ± 0.4	383 ± 46
SCF/EPO	94.0 ± 7.0	31960 ± 2380
SCF/EPO/OSM	23.7 ± 3.9	1824 ± 300
SCF/EPO (+OSM)*	98.0 ± 1.4	-
B.M.	57.7 ± 4.1	-
B.M. (+OSM)*	57.0 ± 1.6	-

Shown are cfu-e activities of hematopoietic cells generated on the fetal hepatic culture. The number of erythroid progenitors in floating hematopoietic cells generated in culture conditions as indicated were determined by the methylcellulose colony formation assay. Cells were incubated for 3 days in the presence of Epo, and the number of erythroid colonies were counted by microscopic observation. As a control, bone marrow-derived hematopoietic cells (B.M.) were similarly tested. Data represent the mean values of triplicate experiments ± SD.

*In these cases, OSM (100 ng/ml) was added during colony assays to exclude the possibility that OSM directly inhibited erythropoiesis.

OSM during co-culture (Table 1), although the total cell number was reduced. However, we observed slight but significant increase of the cfu-mix frequency (per 10^4 cells) in OSM-stimulated condition, suggesting that generation of the lineage-committed progenitors is more susceptible to the OSM effect. In addition, suppression of hematopoiesis is specific to OSM because neither did IL-6, leukemia inhibitory factor, or IL-11 show a similar effect (Fig. 4*A*).

OSM Suppressed Expression of Hematopoietic Factors in Hepatic Stromal Cells. Next, we assessed whether hepatic differentiation alters expression of hematopoietic factors such as cytokines and chemokines in hepatic cells. As shown in Fig. 4 *C* and *D*, OSM inhibited expression of M-CSF and macrophage chemoattractant protein 1 mRNA in hepatic cells (Fig. 4*C*), and chemotactic activity produced in culture media also was decreased by OSM (Fig. 4*D*). Moreover, the OSM receptor is not expressed in $CD45^+$ hematopoietic cells (10), and OSM does not essentially affect colony-forming activities of fetal liver- or bone marrow-derived hematopoietic cells (Table 2; data not shown). These observations strongly suggest that OSM exerts its inhibitory effects by suppressing the hematopoiesis-supporting potential of fetal hepatic cells rather than by directly acting on hematopoietic cells.

Hematopoietic Activity of Hepatic Cells Derived from the E18.5-Embryonic Liver. To further investigate the relationship between fetal liver hematopoiesis and hepatic development, we performed the same experiment using liver cells from E18.5 embryos as a stromal layer. At this stage, hepatocytes have already started expressing a certain set of differentiation markers that can be induced in E14.5-embryonic liver cells by OSM *in vitro*. We added $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ hematopoietic cells derived from E14.5-embryonic liver onto the E18.5 embryo-derived stromal hepatic cells and incubated for 10 days in the presence of SCF. As shown in Fig. 4*B*, E18.5-embryo-derived hepatic cells did not support hematopoiesis, and essentially no floating cells or cobblestone area were found despite the presence of SCF. We therefore conclude that differentiated hepatic cells are no longer capable of expanding or maintaining hematopoietic cells.

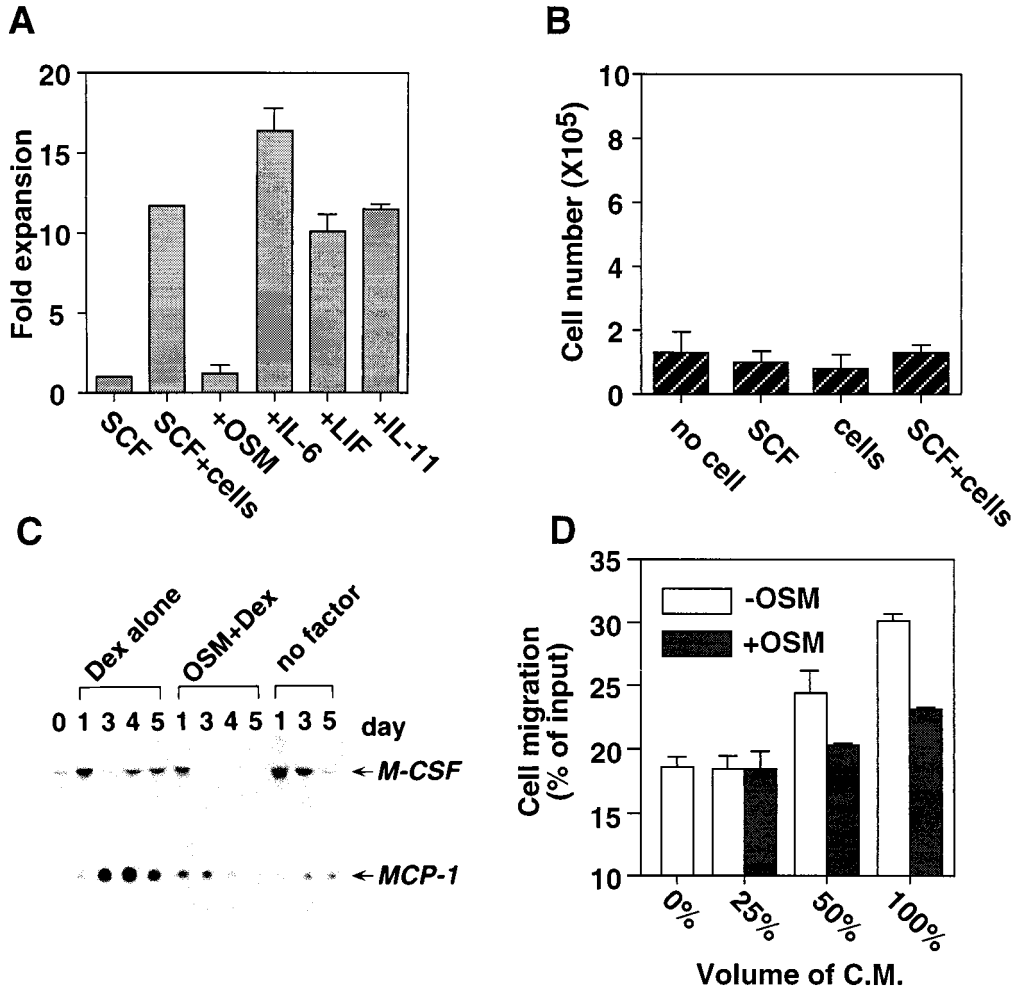

FIG. 4. Characterization of the OSM activity. (*A*) Specificity of hematopoiesis-suppressing activity. $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ cells was co-cultured with hepatic stromal cells as in Fig. 3 in the presence of IL-6-family cytokine. *In vitro* hematopoiesis was inhibited specifically by OSM but not by IL-6, leukemia inhibitory factor, or IL-11. Addition of IL-6 produced 1.5- to ≈2× more floating cells than a control condition because IL-6 is a growth stimulator of hematopoietic cells. (*B*) Failure of the fetal hepatic culture derived from the E18.5-embryonic liver to expand hematopoietic cells from $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ cells, even in the presence of SCF. Each histogram represents the mean value of triplicate experiments ± SD of the absolute number of cells generated in 60-mm-diameter dishes. (*C*) Decrease of mRNA levels for hematopoietic cytokine (M-CSF) and chemokine macrophage chemoattractant protein 1 [(MCP-1)] in fetal hepatic cells in response to OSM and Dex. Fetal hepatic cells were incubated with or without OSM for indicated periods, and total RNA samples were extracted by the acid guanidinium thiocyanate/phenol/chloroform method. Ten micrograms of total RNA samples from cultured cells was analyzed by Northern blotting. The integrity and the amount of RNA samples were evaluated by ethidium bromide staining of the gel (data not shown). (*D*) Decrease of chemotactic activity produced in the conditioned media of the fetal hepatic culture. Various (100, 50, 25, and 0%) concentrations of culture media conditioned either in the presence or absence of OSM for 7 days were applied to an *in vitro* two-chamber migration assay as described in *Materials and Methods*.

DISCUSSION

There are many reports of fetal liver hematopoiesis in which various fetal liver-derived stromal cell lines were shown to support hematopoiesis *in vitro* (19–21). Evidence suggested that distinct populations of stromal cells contribute to different aspects of hematopoiesis, e.g., stem cell maintenance or expansion of lineage-committed cells (21). Those cell lines provide several advantages, particularly in analyzing molecules involved in regulation of hematopoiesis as well as in advancing clinical application of stem cell therapies. However, a microenvironment created by an established cell line(s) is often biased and can be far from the *in vivo* situation. The system developed in this study creates *in vitro* "mini-liver" that can generate myeloid as well as lymphoid cells that contain relatively immature cells, providing a unique system to analyze development of the hematopoietic system and hepatogenesis, which are intimately interacting each other. Indeed, we recently found that not only bone marrow-derived but also AGM region-derived hematopoietic cells are maintained and expanded in this system (M. Takeuchi, T.K., T. Hara, and A.M., unpublished work).

Because promotion of hepatic development by OSM/Dex *in vitro* efficiently attenuated hematopoiesis, it is conceivable that progression of hepatic development *in vivo* toward a metabolically active stage (i.e., the postnatal liver) diminished the capability of hepatic cells to support hematopoiesis. In particular, erythropoiesis was highly susceptible to the inhibitory effect of OSM, even though Epo was sufficiently provided throughout the incubation period. We therefore speculate that hepatic stromal cells play an indispensable role for erythroid differentiation from $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ to the Epo-responsive stage, although a key molecule(s) that is sensitive to the OSM action is yet to be identified. In contrast, HSCs may be less susceptible to hepatic differentiation induced by OSM because the frequency of cfu-mix in floating cells generated in the presence of OSM was slightly higher than those cultured without OSM. Taniguchi *et al.* (22) reported that HSCs are present in the adult liver; therefore, a small population of HSCs may remain in the liver even when most hematopoietic cells have left the liver during development.

We showed evidence that hematopoietic factors expressed by hepatic cells are down-regulated by OSM, confirming that OSM acts directly on hepatic stromal cells and inhibits hematopoiesis. These results also implicate previous reports that several dedifferentiated hepatoma cell lines express hematopoietic cytokines (23, 24). Moreover, regenerating liver was shown to express hematopoietic cytokines (25, 26). Thus, expression of hematopoietic cytokines in the liver appears closely related to the state of liver differentiation, and OSM may be involved in regulation of hematopoietic cytokines in the adult liver as well.

As previously proposed, the bone marrow may attract hematopoietic cells by secreting chemotactic factors to establish adult-type bone marrow hematopoiesis near birth (27–29). Our results suggest another mechanism of transition of hematopoietic cells from the liver to the bone marrow: The developed liver creates a microenvironment unfavorable for hematopoietic cells and thereby excludes the blood cells. Identification of critical molecule(s), in addition to M-CSF and macrophage chemoattractant protein 1, that is regulated by OSM would be an important issue to be addressed, and it also may help us to understand the mechanism of extramedullary hematopoiesis in the adult liver under particular pathologic circumstances.

Based on our observations, we propose a model of liver development as follows (Fig. 5). After the autonomous growth of the liver primodium, definitive HSCs translocate from the AGM region to the liver, where they expand during the midfetal stage. Because hematopoietic cells are producers of

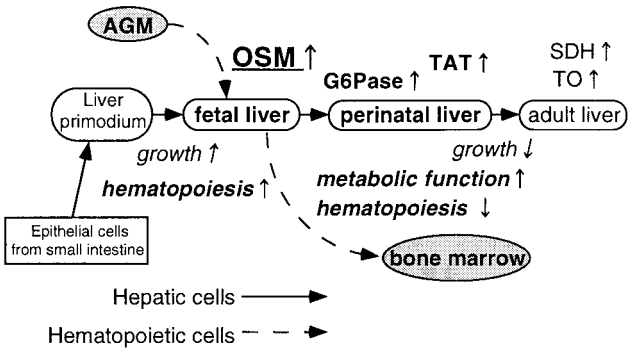

FIG. 5. A possible model for liver development. Hematopoietic cells migrated from the AGM region proliferate in the liver and produce OSM. When the concentration of OSM reaches a threshold level, it stimulates hepatic development in concert with glucocorticoid. As a consequence, the liver loses the capability to support hematopoiesis around the perinatal stage and continues its own maturation programs toward the adult stage. On the other hand, hematopoietic cells migrate to the bone marrow or spleen. TAT, tyrosine aminotransferase; G6Pase, glucose-6-phosphatase; SDH, serine dehydratase; TO, tryptophan oxygenase. All of these enzymes are liver-specific differentiation markers.

OSM, expansion of hematopoietic cells results in the increase of the local OSM concentration, which consequently promotes hepatic development. In response to OSM, hepatocytes begin to acquire liver-specific functions at the expense of hematopoietic activity. When the relationship between hematopoietic cells and hepatic cells terminates, hematopoietic cells relocate to the bone marrow whereas hepatic cells develop toward adult stages (30). In agreement with this hypothesis, preliminary observations using knockout mice deficient for gp130 showed that livers from these mice harbor more hematopoietic cells at perinatal stages than do normal livers (T.K. and A.M., unpublished work). In this scenario, the paracrine action of OSM plays a central role not only in development of the liver but also in the control of embryonic hematopoiesis.

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