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Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer

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Fetal liver, the major site of hematopoiesis during embryonic development, acquires additional various metabolic functions near birth. Although liver development has been characterized biologically as consisting of several distinct steps, the molecular events accompanying this process are just beginning to be characterized. In this study, we have established a novel culture system of fetal murine hepatocytes and investigated factors required for development of hepatocytes. We found that oncostatin M (OSM), an interleukin-6 family cytokine, in combination with glucocorticoid, induced maturation of hepatocytes as evidenced by morphological changes that closely resemble more differentiated hepatocytes, expression of hepatic differentiation markers and intracellular glycogen accumulation. Consistent with these *in vitro* observations, livers from mice deficient for gp130, an OSM receptor subunit, display defects in maturation of hepatocytes. Interestingly, OSM is expressed in CD45$^+$ hematopoietic cells in the developing liver, whereas the OSM receptor is expressed predominantly in hepatocytes. These results suggest a paracrine mechanism of hepatogenesis; blood cells, transiently expanding in the fetal liver, produce OSM to promote development of hepatocytes *in vivo*.
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Introduction

Liver development comprises multiple stages and is influenced by hormonal factors as well as intercellular and matrix–cellular interactions. In mice, the initial event of liver ontogeny occurs on embryonic day 9 (E9); the midgut endoderm commits to become the liver through the interaction with the cardiogenic mesoderm. Then, this liver primodium accepts an inductive signal from the adjacent mesenchym in the heart and invades the septum transversum to give rise to the hepatic codes and bud (Douarin, 1975; Houssaint, 1980). Following these differentiation processes, fetal hepatocytes proceed through a series of maturation steps which accompany autonomous proliferation, cellular enlargement and functional maturation.

The level of hepatic maturation has been characterized by the expression of liver- and stage-specific genes (Derman *et al*., 1981; Panduro *et al*., 1987). Alpha-fetoprotein (AFP) is an early fetal hepatic marker and its expression decreases as the liver develops (Shiojiri *et al*., 1991). In contrast, expression of albumin, the most abundant protein synthesized by hepatocytes, starts in early fetal hepatocytes (E12) and reaches the maximal level in adult hepatocytes (Tilghman and Belayew, 1982). At a late gestation or perinatal stage, hepatocytes start producing a number of metabolic enzymes including glucose-6-phosphatase (G6Pase) and tyrosine aminotransferase (TAT) to prepare their metabolism for the change in the physiological role of the liver (Greengard, 1970; Haber *et al*., 1995). Finally, several days after birth, serine dehydratase (SDH) and tryptophan oxygenase (TO) are induced in hepatocytes (Nagao *et al*., 1986; Noda *et al*., 1990, 1994). Interestingly, expression of these metabolic enzymes is often lost in hepatocytic carcinomas or malignant hepatomas and, conversely, AFP expression resumes in these tumors (Abelev, 1971). Hence, AFP and metabolic enzymes are useful makers to monitor liver development as well as cellular malignancy.

Proliferation and differentiation of hepatocytes are affected by extracellular signals such as hormones and cytokines. For example, glucocorticoid modulates proliferation and function of adult hepatocytes both *in vivo* and *in vitro*. In the fetal liver, physiological concentrations of dexamethasone (Dex), a synthetic glucocorticoid, suppress AFP production and DNA synthesis and up-regulate albumin production (Belanger *et al*., 1981; Nawa *et al*., 1986; de Juan *et al*., 1992). Transforming growth factor-β (TGF-β) is a potent inhibitor of hepatocyte proliferation (Nakamura *et al*., 1985; de Juan *et al*., 1992) and was shown to augment albumin production in prenatal hepatocytes, implicating TGF-β in the regulation of hepatic differentiation (Sanchez *et al*., 1995). Intriguingly, the regulation of TAT mRNA expression depends on the developmental stage of the liver. TAT mRNA, which is virtually absent in the early fetal liver, is induced by Dex in primary hepatocytes of late embryonic stage. In contrast, Dex does not regulate TAT levels at earlier stages (mid-gestation; E12–14), even though these cells are able to express albumin in response to Dex (Shelly *et al*., 1989). These observations suggest that there is a key maturation step between the mid-gestation and late gestation/perinatal

stages. However, the mechanism underlying hepatic development, particularly at the molecular and biochemical level, remains unclear.

Oncostatin M (OSM) is a member of the interleukin-6 (IL-6)-related cytokine family that includes IL-6, IL-11, leukemia inhibitory factor (LIF), ciliary neurotrophic factor and cardiotrophin-1 (Bazan, 1991; Rose and Bruce, 1991; Pennica *et al*., 1995). These cytokines often exhibit similar functions since their receptors utilize gp130 as a common signal transducer (reviewed in Taga and Kishimoto, 1997). In particular, human OSM (hOSM) (Malik *et al*., 1989) shares many biological functions with LIF, such as induction of differentiation in M1 monocytic cells (Rose and Bruce, 1991; Bruce *et al*., 1992) and of acute phase proteins in hepatocytes (Richards *et al*., 1992; Baumann *et al*., 1993). However, hOSM also possesses unique functions, e.g. growth stimulation of endothelial cells (Wijelath *et al*., 1997) and smooth muscle cells (Grove *et al*., 1993). Two types of hOSM receptors have been identified; the type I OSM receptor is identical to the LIF receptor composed of gp130 and the LIF-binding subunit (Gearing *et al*., 1991, 1992; Liu *et al*., 1992), and the type II receptor consists of gp130 and the OSM-specific subunit (Mosley *et al*., 1996). Thus, the common functions of LIF and OSM are mediated by the type I receptor, whereas the unique activities of OSM are transduced by the type II receptor (Thoma *et al*., 1994; Mosley *et al*., 1996). In contrast, mouse OSM utilizes only its specific receptor (the type II receptor) and does not react with the LIF receptor (Ichihara *et al*., 1997; Lindberg *et al*., 1998; Tanaka *et al*., 1999). Mouse OSM was cloned as an IL-3-inducible gene in hematopoietic cells (Yoshimura *et al*., 1996) and was shown to be expressed in various types of hematopoietic cells as well as in the bone marrow (Ichihara *et al*., 1997). Identification of mouse OSM made it possible to analyze OSM-specific actions using murine systems. For example, we recently showed that OSM stimulates generation of definitive hematopoietic cells from putative progenitors (hemangioblasts) which reside in the aorta–gonad–mesonephros (AGM) region of E11 mouse embryo (Mukouyama *et al*., 1998).

Here we describe the establishment of an *in vitro* culture system of fetal hepatic cells and demonstrate that OSM induces development of hepatocytes *in vitro*. Furthermore, studies of gp130 knockout mice reveal an essential role for OSM/gp130 in hepatic maturation *in vivo*. We also show evidence that OSM is produced by hematopoietic cells expanding in the fetal liver. Our results indicate that OSM is a paracrine regulator which plays a pivotal role during fetal liver development.

Results

Morphological changes of fetal hepatocytes in primary culture upon oncostatin M stimulation

In order to investigate the molecular basis of fetal hepatic development, we established a novel primary culture system of murine fetal hepatocytes derived from E14 embryos. Fetal liver cells were dissociated using enzyme-based digestion buffer and plated onto 0.1% gelatin-coated dishes. Several hours later, floating hematopoietic cells and dead cells were removed by extensive washing with

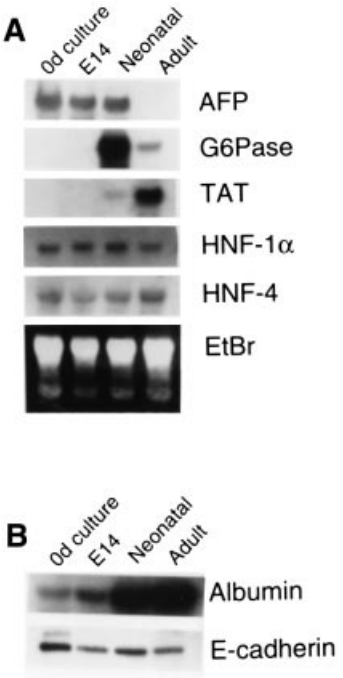

Fig. 1. Characterization of fetal hepatic cells in primary culture. (**A**) Comparison of the expression profiles of liver-specific genes between primary cultured fetal hepatic cells and developing liver tissues. Ten micrograms of total RNAs extracted from fetal hepatic cells free from hematopoietic cells and liver tissues (E14, neonatal and adult) were analyzed by Northern blot using DIG-labeled cDNA probes for AFP, G6Pase, TAT, HNF-1α and HNF-4. Fetal hepatic cells express genes specific for embryonic hepatocytes, while differentiation markers for the postnatal liver were not detected. (**B**) Expression of the albumin and E-cadherin proteins. Protein samples were extracted from fetal hepatic cells and liver tissues (E14, neonatal and adult). Expression of albumin and E-cadherin was examined by Western blot.

culture media. The remaining adhesive cells formed a monolayer sheet (Figure 2A), with the majority of these cells appearing to be hepatocytes as assessed by epithelial morphology. Cultured cells were characterized further by their expression profiles of liver- and stage-specific genes and proteins (Figure 1). mRNAs for the embryonic hepatocyte-specific AFP and for liver-specific hepatic nuclear factors [hepatocyte nuclear factor (HNF)-1 and HNF-4] were expressed in cultured cells. In addition, Western blot analyses showed that cultured cells expressed albumin and E-cadherin proteins, which are present in the E14.5 embryonic liver. On the contrary, cultured cells did not express differentiation markers for the peri- or postnatal liver (G6Pase and TAT). Moreover, these cells grow autonomously without any growth factors such as epidermal growth factor (EGF) and hepatocyte growth factor (HGF) (data not shown). These observations indicate that the majority, if not all, of cultured cells are embryonic hepatocytes that retain many characteristics of fetal hepatocytes *in vivo* at this stage.

Using this system, we searched for a molecule which stimulates hepatic development *in vitro*. Among the various cytokines which we tested, including IL-6, IL-11, LIF, OSM and TGF-β, only OSM showed striking effects on fetal primary hepatocytes. First, addition of OSM resulted in the formation of multiple clusters that closely resemble mature hepatocytes (Figure 2B). These clusters exhibited tight cell–cell contact, highly condensed and

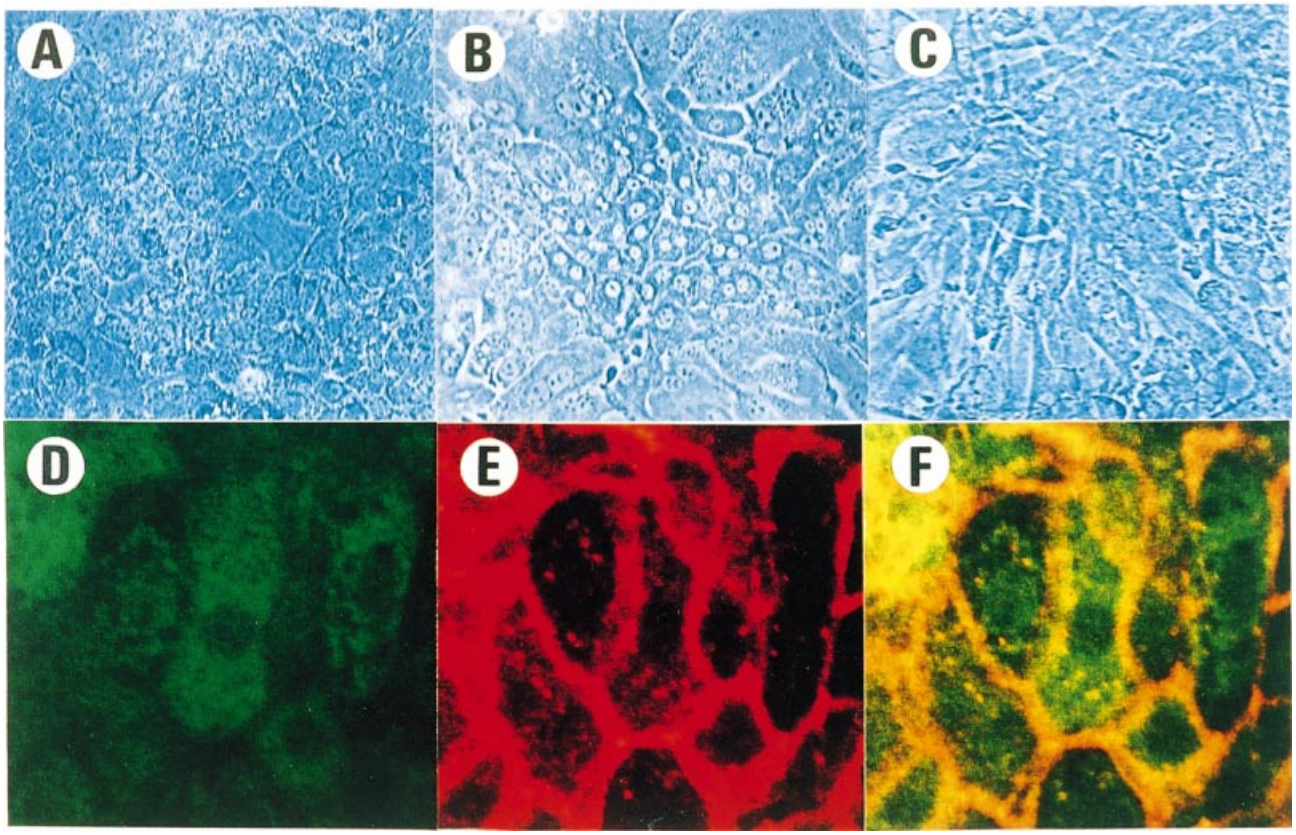

Fig. 2. Morphology of cultured fetal hepatic cells. Fetal hepatic cells derived from the E14 embryonic liver were cultured for 5 days in the absence (A) or presence of 10 ng/ml OSM (B and D–F) or 10 ng/ml TGF-β (C) in combination with glucocorticoid (10^{-7} M dexamethasone). Cultured hepatic cells show a clear epithelial sheet (**A**). OSM induced the formation of multiple clusters that closely resemble more differentiated hepatocytes (**B**). In contrast, TGF-β did not induce such a cluster (**C**). (**D**) Staining of the albumin protein in fetal hepatic cells cultured in the presence of OSM. (**E**) E-cadherin staining of the cells shown in (D). The E-cadherin protein was localized to the sites of homophilic cell adhesion. (**F**) Superimposition of (D) and (E), showing that cell clusters are positive for both albumin and E-cadherin.

granulated cytosol and clear round-shaped nuclei in a way similar to mature hepatocytes. To examine whether these cell clusters were differentiated hepatocytes, we stained them with anti-E-cadherin and anti-albumin antibodies. As shown in Figure 2E, E-cadherin was localized to the periphery of cells that constitute clusters induced by OSM, although the total E-cadherin level did not change before or after OSM stimulation (data not shown). Co-localization of E-cadherin and albumin in the same cells revealed that E-cadherin^{+} cell clusters are parenchymal hepatocytes (Figure 2D and F). We also found that the presence of a physiological concentration of glucocorticoid (10^{-7} M Dex) is absolutely required for the OSM action; addition of OSM (up to 100 ng/ml) failed to induce the cluster formation in the absence of Dex (data not shown). These results indicate that OSM and glucocorticoid collaborate to induce morphological changes in fetal hepatocytes.

Regulation of albumin production by OSM and Dex

E14 embryonic liver expresses a detectable amount of albumin, although the level of expression was significantly lower than that in adult hepatocytes (Figure 1B). Since albumin production is an important and characteristic liver-specific function, we examined whether OSM would regulate albumin production in cultured hepatocytes. After 6 days of incubation with or without cytokines, equal amounts of total cellular proteins were examined for albumin production by Western blot analysis. When fetal hepatocytes were cultured in the absence of exogenously added factors, albumin production gradually decreased to a low level within 2–3 days as shown in Figure 3A, consistent with the previous observation (Sanchez *et al*., 1995). In contrast, albumin production was maintained in the presence of both 10 ng/ml OSM and 1.0×10^{-7} M Dex (Figure 3A). The effect was dose dependent, ranging from 0 to 10 ng/ml for OSM and from 0 to 10^{-6} M for Dex (Figure 3B and C). Again, the presence of both factors was necessary to maintain albumin production. TGF-β was shown previously to maintain the albumin level in cultured hepatocytes derived from E21 rat embryos (Sanchez *et al*., 1995). However, no such effect was observed in E14 mouse embryo-derived hepatocytes in our system. Probably, E14 embryo-derived hepatocytes are less mature and therefore not sensitive to TGF-β stimulation for the production of albumin.

Gene induction of hepatic differentiation markers in vitro by OSM and Dex

During hepatic development, expression of liver-specific genes is strictly regulated in accordance with the requirement for stage-specific liver functions (Panduro *et al*., 1987). Striking changes in gene expression occur around the perinatal stage, since the liver needs to assume metabolic functions soon after birth. In rats, for example, G6Pase begins to be produced at E20–21 (just before

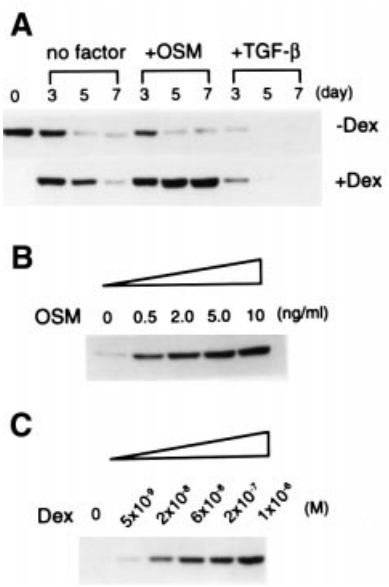

Fig. 3. Regulation of albumin production by OSM and Dex *in vitro*. (**A**) Time course of albumin expression during primary culture in the presence of various combination of OSM (10 ng/ml), TGF-β (10 ng/ml) and Dex (10^{-7} M). (B and C) Dose dependence of albumin production on OSM in the presence of Dex (10^{-7} M) (**B**) or on Dex in the presence of OSM (10 ng/ml) (**C**). After a 7 day incubation, protein samples were extracted from hepatocytes and albumin production was examined by Western blot using the anti-albumin antibody.

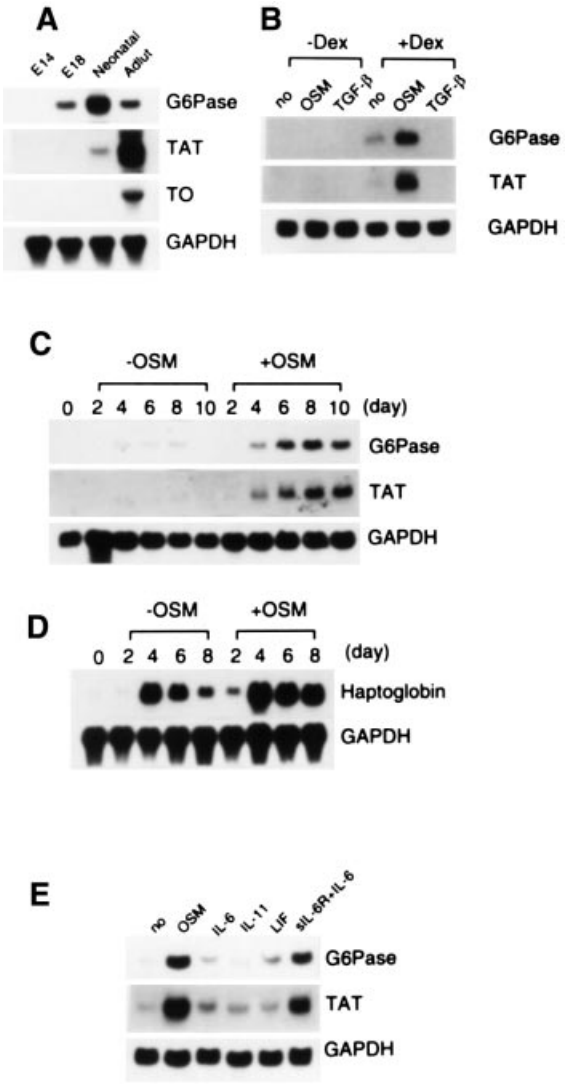

Fig. 4. Expression of differentiation markers in response to OSM (10 ng/ml) and Dex (10^{-7} M). (**A**) Developmental changes in expression patterns of liver-specific differentiation markers *in vivo*. Ten micrograms of total RNAs from E14, E18, neonatal and adult liver tissues were analyzed by Northern blot using DIG-labeled cDNA probes for G6Pase, TAT, TO and GAPDH (internal control). (**B**) Induction of mRNA for hepatic differentiation markers *in vitro* by OSM and Dex. (**C**) Time courses of G6Pase and TAT mRNA expression after OSM stimulation. (**D**) Time course of induction of haptoglobin mRNA after OSM stimulation. (**E**) Failure of other IL-6-related cytokines to induce G6Pase or TAT mRNA expression.

birth) and its expression reaches the maximum at 1 h after birth; likewise, TAT mRNA first appears in the postnatal liver and further increases with maturation. Similar expression profiles of mRNA for G6Pase and TAT were observed for murine hepatic development (Figure 4A). Thus, these genes are also useful makers to monitor the level of hepatic maturation in mice.

To examine whether mRNA expression of G6Pase and/or TAT is inducible by OSM and Dex, fetal hepatic cultures were stimulated with either OSM or TGF-β (Figure 4B). In the absence of Dex, no expression of either mRNA was detected even when OSM was added. Stimulation by Dex alone induced G6Pase mRNA only slightly around days 4–8, and the expression decreased thereafter. In contrast, hepatocytes, cultured with both OSM and Dex, started expressing G6Pase mRNA from 4 days after stimulation, and the expression level continued to increase to reach a maximum after 8–10 days (Figure 4C). Induction of TAT mRNA expression was even more remarkable. OSM induced TAT mRNA expression after 4 days of culture, with a maximum obtained on day 8. As TAT mRNA was barely detectable in the absence of OSM, *in vitro* TAT induction strictly depends on OSM. In contrast, mRNA for an acute phase protein (haptoglobin) was induced more quickly by OSM than those for G6Pase and TAT (Figure 4D). These results suggest that molecular mechanisms underlying the induction of differentiation markers and acute phase proteins (Richards *et al*., 1992, 1997) are different, at least in part. Other IL-6-related cytokines (IL-6, LIF or IL-11) failed to stimulate induction of these enzymes; however, IL-6, when combined with the soluble IL-6 receptor (Yasukawa *et al*., 1992; Yawata *et al*., 1993), induced comparable levels of mRNA for both enzymes (Figure 4E). This suggests that the lack of an IL-6 response is due to lack of IL-6Rα expression and that OSM transduces differentiation signals through the common signaling receptor subunit, gp130.

The continuous presence of OSM is necessary for induction of differentiation markers

To determine the window of time during which OSM is required for hepatic maturation, we tested the effects of removal and delayed addition of OSM to the culture on *in vitro* maturation of fetal hepatocytes (Figure 5). Hepatocytes were first stimulated with OSM/Dex and then OSM was removed at various time points as indicated in the figure. Expression of differentiation markers was analyzed on day 7 post-stimulation. Figure 5A shows that removal of OSM before day 5 reduced the induction of differentiation markers. On the other hand, when OSM

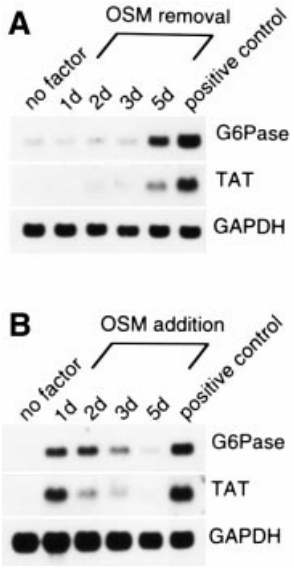

Fig. 5. Continuous OSM stimulation is required for the *in vitro* hepatic maturation process. (**A**) The effect of OSM removal on gene activation of hepatic differentiation markers. Fetal hepatocytes were stimulated with OSM on day 0, and OSM was then removed from culture media on day 1, 2, 3 or 5 as indicated. Gene expression was examined on day 7. (**B**) OSM was added on day 1, 2, 3 or 5 after plating, and gene expression was examined on day 7.

was removed on day 5, expression of differentiation markers was induced although the induction levels were slightly lower than those supported by continuous OSM stimulation. Conversely, delaying the addition of OSM reduced expression of differentiation markers; the later the addition of OSM, the lower the observed level of mRNA expression (Figure 5B). It is therefore likely that the induction of hepatic maturation *in vitro* requires continuous OSM stimulation.

Stimulation of glycogenesis in vitro by OSM and Dex

Regulation of the blood glucose level is another important function of the differentiated liver, and this is controlled by the rate of glycogenesis and glycogen breakdown (Nemeth *et al*., 1953; Foster *et al*., 1966). Glycogenesis first occurs during late fetal development, and both perinatal and more differentiated hepatocytes store a large reserve of glycogen (Yeung and Oliver, 1967; Philippidis and Ballard, 1969). To examine whether OSM/Dex induce functional maturation of hepatocytes to produce and store glycogen, we analyzed accumulation of intracellular glycogen *in vitro* by the Periodic acid–Schiff (PAS) staining method. Fetal hepatic cells cultured for 2 or 6 days in the presence of OSM were stained with the PAS reagent. No storage of glycogen was detected in cells incubated for 2 days regardless of the presence of OSM and/or Dex

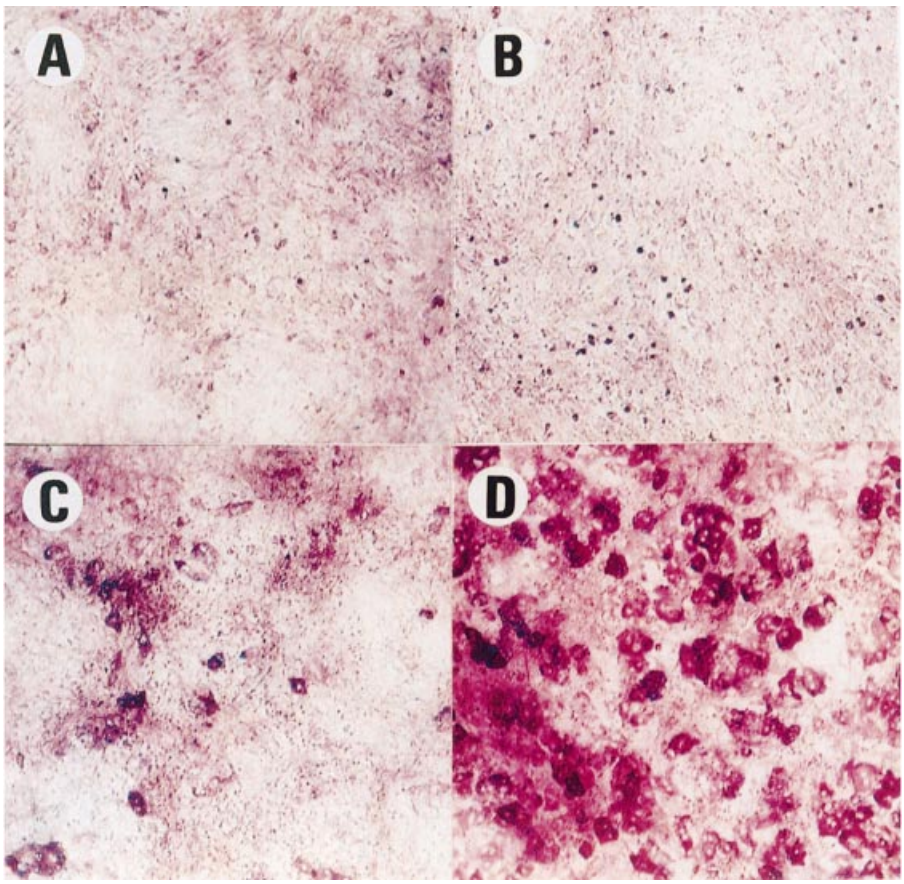

Fig. 6. Glycogenesis in cultured hepatocytes. Cultured fetal hepatocytes were incubated either with no factor (**A**), OSM alone (**B**), Dex alone (**C**) or OSM plus Dex (**D**). Cells were then stained with the PAS solution as described in Materials and methods. A number of PAS-positive cells appeared when stimulated with both OSM and Dex for 6 days.

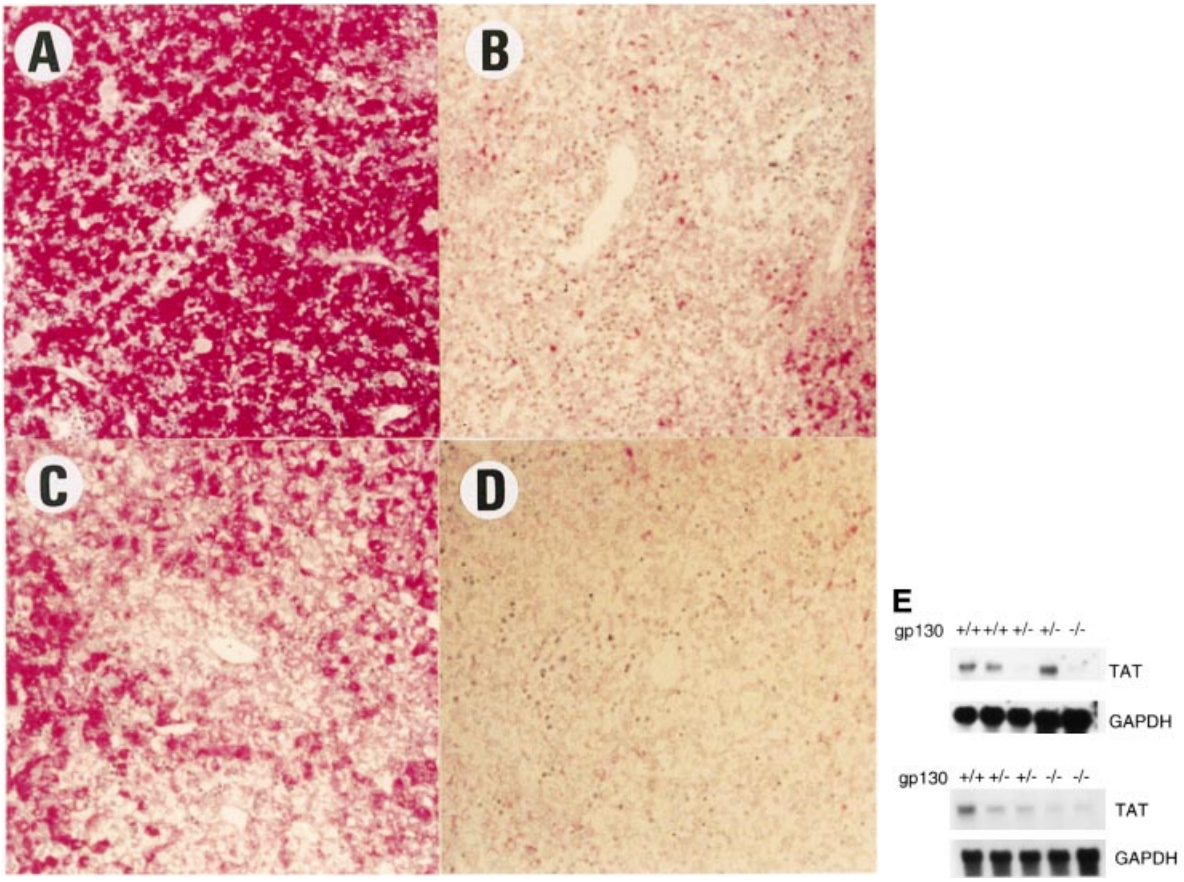

Fig. 7. Defective maturation of the liver from gp130 knockout mice. (A–D) Glycogen storage in gp130$^{-/-}$ or control livers. Liver tissues from the wild-type (**A** and **C**) or gp130$^{-/-}$ mice (**B** and **D**) at E17 (A and B) or at a neonatal stage (C and D) were stained with the PAS solution. PAS staining of gp130$^{-/-}$ livers was much weaker than those of control mice at both stages. (**E**) Expression of a hepatic differentiation marker in gp130$^{-/-}$ and wild-type mice. mRNA samples were prepared from E17 embryonic livers and expression of TAT mRNA was examined by Northern blot. The level of TAT mRNA was significantly reduced in knockout livers. In some cases, gp130$^{+/-}$ mice showed a reduced level of TAT mRNA expression. This is consistent with the previous observation that gp130$^{+/-}$ mice show heterogeneous phenotypes in different assays (Yoshida *et al.*, 1996).

(data not shown). In contrast, after 6 days of incubation, we noted slight accumulation of glycogen in some hepatocytes cultured with Dex alone, and OSM/Dex strongly induced glycogen accumulation in the majority of the cells (Figure 6C and D). On the other hand, cells cultured with OSM alone or in the absence of both factors did not store a significant amount of glycogen even on day 6 (Figure 6A and B). These results indicate that, in addition to the morphological changes and induction of differentiation markers, OSM/Dex induce functional maturation of hepatocytes.

Defective development of the liver in gp130 knockout mice

The results described above strongly suggest the importance of OSM and its receptor in hepatic development. To study further the role of the OSM/OSM receptor (OSMR) system *in vivo*, we analyzed gp130$^{-/-}$ mice. Although gp130 deficiency originally was described to be lethal around E14 in C57BL/6 mice (Yoshida *et al.*, 1996), gp130$^{-/-}$ mice of the ICR genetic background often survive longer and die soon after birth (Kawasaki *et al.*, 1997). These mice thus enabled us to analyze late fetal liver development. To investigate the liver function in gp130$^{-/-}$ mice, glycogen accumulation in the liver was examined. As shown in Figure 7, normal hepatocytes store a large amount of glycogen at both E17 and new-born stages (Figure 7A and C). In contrast, accumulation of intracellular glycogen was greatly reduced in the liver from gp130$^{-/-}$ mice (Figure 7B and D), although there were some PAS-positive cells along the periphery of liver lobules. This limited accumulation of glycogen in the gp130$^{-/-}$ liver was comparable with the small number of PAS-positive cells when fetal hepatocytes were cultured with Dex alone (Figure 6C). This suggests that although glucocorticoid induces limited glycogen accumulation, full metabolic function requires the OSM–gp130 signaling pathway. Defects found in gp130$^{-/-}$ mice are reminiscent of those in C/EBPα knockout mice (Wang *et al.*, 1995). In C/EBPα$^{-/-}$ mice, hepatocytes are negative for PAS, although effects on differentiation markers are relatively modest. Likewise, TAT expression was reduced to some extent in the liver from gp130$^{-/-}$ mice (Figure 7E). Thus, while expression of these genes can be partly controlled by glucocorticoid or unknown factors, development of a functional liver appears to require the presence of both gp130 and C/EBPα signaling pathways.

Expression of OSM and OSM receptor mRNAs in the developing liver

Induction of differentiation markers, as well as the morphological and functional maturation specifically elicited by

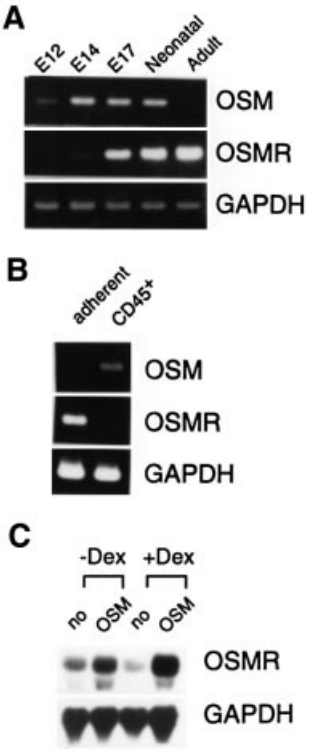

Fig. 8. Expression of OSM and OSMR *in vivo*. (**A**) RT–PCR analysis of OSM and OSMR mRNAs in liver tissues during development. (**B**) Localization of OSM and OSMR mRNAs in different cell populations in the E14 embryonic liver. mRNA samples prepared from $CD45^+$ hematopoietic cells and adherent hepatocytes free from hematopoietic cells were analyzed by RT–PCR analysis. OSM mRNA was produced specifically by $CD45^+$ hematopoietic cells, whereas OSMR mRNA was expressed mainly by adherent hepatocytes. (**C**) Induction of OSMR mRNA by OSM *in vitro*. Cultured cells were stimulated with OSM (10 ng/ml) with or without Dex (10^{-7} M) for 7 days, and mRNA for OSMR was analyzed by Northern blot.

OSM/Dex, suggest that these molecules are involved in hepatic ontogeny *in vivo*. While glucocorticoid presumably is provided through the blood circulation, a cytokine such as OSM is often produced locally at the site of action. Accordingly, it is likely that both OSM and OSMR co-exist in the developing liver, if the OSM/OSMR system is really involved in the maturation process *in vivo*. We therefore investigated expression of OSM and OSMR mRNAs in developing liver tissues (Figure 8). OSM mRNA was clearly detected in the liver, starting from E12 through the neonatal stage. On the other hand, OSMR mRNA expression became apparent from E14 and continued to be expressed in the adult liver (Figure 8A). Next, we separated E14 embryonic liver cells into $CD45^+$ hematopoietic cells and adherent cells and examined the expression of OSM and OSMR mRNAs in each population. Interestingly, OSM mRNA was expressed specifically in $CD45^+$ hematopoietic cells and OSMR mRNA was found in CD45-adherent cells which are predominantly hepatocytes (Figure 8B). Thus, OSM is likely to be a hematopoietic cell-derived paracrine factor that acts on neighboring hepatocytes. In addition, expression of OSMR *in vitro* was up-regulated by OSM stimulation, suggesting a positive feedback mechanism in fetal hepatocytes (Figure 8C).

Discussion

During development, the liver performs a number of stage-specific biological functions ranging from the support of hematopoiesis in the fetus (Johnson and Moore, 1975) to highly sophisticated metabolic functions throughout life. The metabolic functions of the liver are divided further into fetal, neonatal and adult stages, which reflect and are necessitated by differences in the source of nutrition. Therefore, there are critical maturation steps toward adulthood during which hepatocytes prepare for a new phase of life. The transition from the mid- or late fetal to the neonatal stage is likely to be particularly drastic since the metabolism of new-born animals must be totally independent of their mother's systems. In order to prepare for this change, hepatocytes begin to express molecules such as G6Pase and TAT before or near birth; however, the precise molecular mechanism of this process remains unknown, and no soluble factor regulating this process has been documented except for glucocorticoid hormones.

In this study, we have established a primary culture system of murine fetal hepatocytes to investigate the mechanism that controls late fetal liver development. Cultured fetal liver cells derived from E14 mouse embryos retain many characteristics of *in vivo* hepatocytes at this stage. Using this system, we demonstrated that OSM in concert with glucocorticoid specifically induces many characteristics of more differentiated hepatocytes: the morphology of differentiated hepatocytes, induction of differentiation markers for the postnatal liver and functional maturation (i.e. glycogenesis). None of the other IL-6-related cytokines (IL-6, LIF and IL-11) nor TGF-β induced these differentiation markers (Figure 4). Notably, however, IL-6 exerted a comparable activity when combined with the soluble IL-6 receptor; therefore, the effect of OSM is mediated by the signal transducer gp130. It is also noteworthy that the presence of glucocorticoid was indispensable for induction by OSM of differentiated phenotypes in fetal hepatic cells (Figures 4B and 6B). In agreement with these data, expression of hepatic differentiation markers is impaired in mice deficient for the glucocorticoid receptor (Cole *et al*., 1995). These results indicate that glucocorticoid plays an essential role in hepatic development. However, as suggested by Sassi *et al*. (1998), glucocorticoids are not sufficient for hepatic development and, in fact, stimulation by Dex alone elicited only a limited sign of hepatic maturation *in vitro* (Figures 4 and 6; Shelly *et al*., 1989). Therefore, it is likely that full stimulation of hepatic development requires the signal from OSM/OSMR.

Knockout mice deficient for gp130, an OSM receptor subunit, showed defective development of hepatocytes; failure of glycogen storage and reduced expression of TAT mRNA. We noted, in some cases, that the TAT mRNA level was reduced to some extent in the $gp130^{+/-}$ liver, correlating with intermediate phenotypes of $gp130^{+/-}$ mice observed in several other assays (Yoshida *et al*., 1996). Therefore, we conclude that gp130 is implicated in the process of hepatic maturation *in vivo*. Interestingly, the phenotypes of the liver from $gp130^{-/-}$ mice were similar to those in $C/EBP\alpha^{-/-}$ mice (Wang *et al*., 1995). C/EBPα is a transcription factor, and the 5′-transcriptional regulatory region of the TAT gene contains the target

sequence of C/EBPα. It is therefore possible that signaling pathways activated by gp130 cooperate with the C/EBPα signal directly or indirectly. Recently, Burgess-Beusse and Darlington (1998) reported that the activation of STAT3 during the acute phase response was deficient in the liver of C/EBPα$^{-/-}$ mice. Analysis of intracellular signaling pathways downstream of OSM/gp130 would be an interesting subject to be addressed.

We provide the first evidence that hematopoietic cells expanding in the embryonic liver play an important role in stimulating hepatic development by producing OSM. Hematopoietic stem cells (HSCs) originating from the AGM region or possibly the yolk sac migrate to the fetal liver around the E11 stage. HSCs then expand in the hepatic microenvironment and generate numerous HSCs as well as lineage-committed cells. Since hematopoietic cells produce OSM, expansion of hematopoietic cells results in the increase of the local OSM concentration which consequently promotes hepatic development. Furthermore, we recently found that our culture system is capable of supporting hematopoiesis *in vitro*. Interestingly, such an intimate relationship between hepatic cells and hematopoietic cells is lost during OSM-induced hepatic maturation (T.Kinoshita *et al*., submitted). Paracrine regulation of the parenchymal liver occurs not only during hepatic development but also in liver regeneration, although the paracrine partner for hepatocytes may be different. One important factor triggering hepatic regeneration is HGF (reviewed in Matsumoto and Nakamura, 1997) which is produced in non-parenchymal liver cells (Kinoshita *et al*., 1989), possibly sinusoidal endothelial cells and Ito cells, in response to hepatic insults (Noji *et al*., 1990; Ramadori *et al*., 1992). In turn, hepatocytes produce vascular endothelial growth factor to promote proliferation of endothelial cells (Yamane *et al*., 1994). In the rat system, expression of HGF in the liver becomes apparent several days after birth (Kagoshima *et al*., 1992; Hu *et al*., 1993); therefore, HGF produced by non-parenchymal liver cells may be involved in postnatal development of the liver. IL-6 is also a key regulator of liver regeneration (reviewed in Michalopoulos and DeFrances, 1997), and IL-6 knockout resulted in the loss of a regenerative response of hepatocytes after hepatic damage (Cressman *et al*., 1996). In this case, Kupffer cells are responsible for production of IL-6. Thus, close relationships between different cell types appear to be a central mechanism regulating the liver modeling and remodeling.

Liver development comprises multiple stages. As previously reported, the initial formation of the liver primodium requires c-Jun and SEK1 (Hilberg *et al*., 1993; Nishina *et al*., 1999). The presence of hepatocytes in gp130$^{-/-}$ mice suggests that IL-6-related cytokines are not absolutely necessary for this process. Thus, distinct sets of molecules appear to be involved in different stages of liver development. Here, we have demonstrated that liver development from the mid-fetal to neonatal stages requires OSM/gp130. However, it may not be the only mechanism of late fetal liver development and there may be another level of regulation. One such example is insulin, which is considered to suppress maturation of hepatocytes *in utero* (Ho *et al.*, 1981; Cake, 1986), although incubation of fetal hepatocytes without insulin does not allow cells to express differentiation markers coordinately (data not shown). Perhaps both the OSM/gp130 system and such signals are functioning *in vivo*. Further analysis using our culture system as well as gp130$^{-/-}$ mice will help us to extend our understanding of the mechanism of hepatic development.

Materials and methods

Materials

C57BL/6CrSlc mice (Nihon SLC, Japan) were used in all experiments in this study. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), liver perfusion medium, liver digest medium, minimal essential medium (MEM) non-essential amino acid solution and insulin–transferrin–selenium X (ITS) were purchased from Gibco-BRL. Human IL-6, human IL-11, murine LIF and human TGF-β were kindly provided by Ajinomoto Co. and KIRIN Brewery (Japan). Murine OSM was from R&D Systems. Rabbit polyclonal anti-mouse albumin antibody was from Nordic Immunological Laboratories (The Netherlands) and mouse monoclonal anti-human E-cadherin antibody was from Transduction Lab. (USA). PCR primers for OSM, TAT, G6Pase, HNF-1α and HNF-4 were synthesized based on reported sequences. Murine OSMR primers were synthesized based on the sequence of the cDNA clone that we described recently (Tanaka *et al*., 1999).

Cell culture

Minced embryonic liver tissues from C57BL/6CrSlc mice (E14.5) were dissociated with enzyme-based dissociation buffer (liver digest medium) followed by hemolysis with hypotonic buffer. Dissociated cells were suspended in culture media composed of DMEM supplemented with 10% FCS, 2 mM L-glutamine, 1× non-essential amino acid solution, 1× ITS, 50 μg/ml gentamycin and 10^{-7} M Dex and plated onto 0.1% gelatin-coated tissue culture dishes. Several hours later, contaminating hematopoietic cells and cell debris were removed by extensive washing with culture media. Culture media were replaced every 2 days.

Immunofluorescence analysis

Cultured fetal liver cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100 for 15 min. Cells were then incubated for 1 h with both the anti-albumin antibody and the anti-E-cadherin antibody. Samples were washed with phosphate-buffered saline (PBS) and incubated with the secondary antibodies, anti-rabbit Ig, fluorescein-linked antibody (Boehringer Mannheim) and anti-mouse Ig, phycoerythrin-linked antibody (Leinco Tech. Inc., UK). Confocal analysis was performed with a confocal laser scanning microscope (Leika Instruments, Germany).

Analysis of albumin production by Western blot

Detergent lysates of cytokine-stimulated or non-stimulated cells were prepared by lysing cells in NP-40 lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris–HCl pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 mM sodium vanadate and 1 mM EDTA). The soluble fraction of each sample was resolved under reducing conditions by SDS–PAGE prior to transfer to Immobilon™ membranes (Millipore). The albumin protein was detected on Western blots using 1 μg/ml anti-mouse albumin antibody diluted in Tris-buffered saline (TBS) containing 3% bovine serum albumin (BSA) followed by a horseradish peroxidase-conjugated anti-rabbit IgG (Amersham). Immunoreactive bands were developed by ECL (Amersham) and blots were exposed to Bio-Max film (Kodak).

Analysis of mRNA expression by Northern blot

Cellular and tissue mRNA samples were purified by the acid guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987). Ten micrograms of total RNA of each sample was separated by electrophoresis on 1.5% agarose gels containing 2% formaldehyde and transferred to positively charged nylon membranes (Boehringer Mannheim). After UV irradiation, membranes were hybridized in high SDS buffer [7% SDS, 50% formamide, 5× SSC, 2% blocking reagent (Boehringer Mannheim), 50 mM sodium phosphate (pH 7.0), 0.1% *N*-lauroylsarcosine] with digoxigenin (DIG)-labeled cDNA probes generated by the r*Taq* DNA polymerase reaction. Blots were then treated with alkaline phosphatase-labeled anti-DIG antibody (Boehringer Mannheim) and developed with CDP-star (New England Biolabs) according to the manufacturer's instructions.

mRNA detection by RT–PCR

$CD45^+$ cells were separated from E14 embryonic liver cell suspension by the anti-mouse CD45 antibody (Pharmingen) and Dynabeads M-450 anti-Rat IgG (Dynal A. S., Norway). First-strand cDNA was synthesized from $CD45^+$ or adherent hepatic cells using the First-Strand cDNA Synthesis Kit (Pharmacia). Synthesized cDNA samples were used as templates for PCR amplification of murine OSM or OSMR. Primer annealing was performed at 50°C for 60 s and amplification was carried out for 35 cycles. The amplified products were separated by electrophoresis on 1.5% agarose gels and stained with ethidium bromide.

PAS staining analysis of cultured cells and tissue sections

Fetal hepatocytes cultured under various conditions or paraffin-embedded sections of liver tissues were fixed in 20% formaldehyde, and intracellular glycogen was stained with the PAS staining solution (Muto Pure Chem., Japan) according to the standard protocol (Nettleton and Carpenter, 1977).

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