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Expression and function of c-Kit in fetal hemopoietic progenitor cells: transition from the early c-Kit-independent to the late c-Kit-dependent wave of hemopoiesis in the murine embryo

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SUMMARY

The protooncogene c-*kit* encodes a receptor type tyrosine kinase and is allelic with the *W* locus of mice. SLF, the c-Kit ligand which is encoded by the *Sl* locus, has growth promoting activity for hemopoietic stem cells. Previous studies demonstrated that c-Kit is functionally required for the proliferation of hemopoietic progenitor cells at various differentiation stages in adult bone marrow. However, the absence of functional SLF and c-Kit in fetuses with mutant alleles of *Sl* and *W* loci produces only minor effects on the myeloid and early erythroid progenitor cells in the fetal liver, although the level of the late erythroid progenitor cells is significantly affected. We used an anti-c-Kit monoclonal antibody to investigate the expression and function of c-Kit in murine fetal hemopoietic progenitor cells. Flow-cytometric analysis showed that hemopoiesis in the yolk sac and fetal liver started from cells that express c-Kit. The c-Kit expression decreased upon maturation into erythrocytes in each organ. By fluorescence activated cell sorting, the c-Kit$^+$ cell population was enriched with the hemopoietic progenitor cells clonable in vitro (CFU-E, BFU-E and GM-CFC). To elucidate whether c-Kit functions in these progenitor cells in vivo, we took advantage of the antagonistic anti-c-Kit monoclonal antibody, ACK2, which can block the function of c-Kit. Administration of ACK2 after 12.5 days of gestation rapidly eliminated BFU-E and GM-CFC as well as CFU-E from the fetal liver. However, the number of these progenitor cells in the yolk sac and fetal liver was less affected when the fetuses were given ACK2 before 12.5 days of gestation. Our results provide evidence that there are two waves of hemopoiesis in murine embryos relative to c-Kit dependency. The c-Kit has an essential role on the growth of hemopoietic progenitor cells in the fetal liver after 12.5 days of gestation, whereas the progenitor cells in the liver and yolk sac of the earlier embryo do not depend on c-Kit and its ligand SLF.

Key words: c-*kit*, *steel*, hematopoietic stem cell, fetal hematopoiesis, monoclonal antibody

INTRODUCTION

The major site of hemopoiesis changes during the ontogeny of many vertebrate species. In the mouse embryo, hemopoiesis begins in the yolk sac mesoderm by 7.5 days of gestation. Large nucleated primitive erythrocytes and the circulatory system develop in the blood islands simultaneously, thereby recruiting the primitive erythrocytes into the systemic circulation at 9 days of gestation. While the yolk sac continues to generate blood cells until 13 days of gestation, the major hemopoietic site shifts to the fetal liver at 10 days of gestation, where definitive erythrocytes containing adult forms of hemoglobin, myeloid and lymphoid cells first appear (Barker, 1968). The site of hemopoiesis further shifts from the fetal liver to the spleen at a later gestational stage and eventually settles in the ultimate destination, the bone marrow (Burgess and Nicola, 1983).

The most important issue yet to be resolved is whether these successive waves of hemopoiesis taking place in dif-

ferent organs reflect an orderly migration of the stem cells that developed in the yolk sac or whether each wave is mediated by the stem cells generated de novo in each organ. Previous studies have demonstrated that the hemopoietic progenitor cells that form colonies in the spleen of the lethally irradiated mouse or in semi-solid culture, develop first in the yolk sac and migrate to the fetal liver (Moore and Metcalf, 1970; Perah and Feldman, 1977; Wong et al., 1986; Hollands, 1987). However, other investigators have detected progenitors of T or B lymphocytes first in the embryonal body rather than in the yolk sac (Tyan and Herzenberg, 1968; Ogawa et al., 1988). In the chick, each wave of embryonal hemopoiesis is mediated by stem cells that developed in separate locations. For example, the hemopoietic stem cells that are responsible for hemopoiesis in adult life originate from an intra-embryonic source rather than the yolk sac, probably in the cluster of hemopoietic progenitors in the dorsal aorta (Dieterlen-Lièvre, 1975; Cormier and Dieterlen-Lièvre, 1988). An aortic cell cluster similar to that seen in the avian embryo has been described in the mouse embryo at 10 days of gestation (Smith and Glomski, 1982).

To address this question, we need to understand the molecular basis underlying the self-renewal and migration of fetal hemopoietic stem cells. In recent years, remarkable progress has been made in elucidating the molecular requirements for the proliferation of hemopoietic progenitor cells. Among these, KL/mast cell growth factor/stem cell factor (SLF) has been established as an essential molecule for the self-renewal of hemopoietic stem cells by phenotype analysis of the mouse. Mutations at the *dominant spotting* (*W*) locus cause developmental defects of melanocytes, germ cells and hemopoietic cells. Virtually identical symptoms are also detected in the mouse with mutations at the *steel* (*Sl*) locus (Russell, 1979; Kitamura, 1989). Recently, the *W* and *Sl* locus have been mapped to the genes encoding the receptor tyrosine kinase c-Kit and its ligand SLF, respectively (Chabot et al., 1988; Geissler et al., 1988; Nocka et al., 1990; Williams et al., 1990; Copeland et al., 1990; Flanagan and Leder, 1990; Zsebo et al., 1990b; Huang et al., 1990). Studies using a recombinant form of SLF in combination with other growth factors showed that it has indeed growth promoting activity for multipotent hemopoietic progenitor cells (Anderson et al., 1990; Zsebo et al., 1990a; Martin et al., 1990; Tsuji et al., 1991; Metcalf and Nicola, 1991; Broxmeyer et al., 1991; Migliaccio et al., 1991; de Vries et al., 1991; Bodine et al., 1992). Moreover, administration of the antagonistic anti-c-Kit monoclonal antibody to the adult mouse induced a severe reduction in the number of hemopoietic progenitor cells followed by depletion of mature myeloid and erythroid cells from the bone marrow (Ogawa et al., 1991).

All these results unequivocally indicate that the self-renewal of immature hemopoietic progenitor cells in the adult bone marrow is dependent upon SLF. However, the role of SLF in fetal hemopoiesis is yet to be determined. Because the anemia of *W/W* mouse and *Sl/Sl*^{d} mouse is detectable at 12 and 13 days of gestation, respectively, c-Kit and the ligand play an essential role in fetal erythropoiesis (Russell et al., 1968; Chui and Russell, 1974; Chui and Loyer, 1975). On the other hand, it was also shown that the levels of burst forming unit-erythroid (BFU-E) and granulocyte/macrophage-colony forming cells (GM-CFC) are normal even in the fetal liver of *W/W* mouse, which cannot express functional c-Kit, although the level of colony forming unit-erythroid (CFU-E) was markedly reduced (Nocka et al., 1989). These observations would suggest that the c-Kit functions in proliferation of the late but not the early erythroid progenitors and the myeloid progenitors in fetal liver. Alternatively, it is possible that the self-renewal of most of the hemopoietic progenitors in the fetal liver is dependent upon c-Kit as in the adult bone marrow, while the c-Kit function is compensatable in the early erythroid and myeloid progenitors. To resolve this issue, the monoclonal antibody-mediated suppression of c-Kit function has a considerable advantage over molecular genetics, since the timing of suppression can be controlled. In the present study, we investigated the expression and the function of c-Kit in fetal hemopoietic progenitors. Our results demonstrate that c-Kit is expressed on most of the hemopoietic progenitors regardless of embryonic age or site. The c-Kit is requisite to fetal granulopoiesis as well as erythropoiesis after 12.5 days of gestation, whereas the hemopoietic progenitors in the earlier fetal liver and yolk sac are less dependent upon c-Kit function.

MATERIALS AND METHODS

Mice

Female and male BALB/c mice purchased from Japan SLC Inc. (Shizuoka, Japan) were mated from 6 p.m. to 9 a.m. The embryos were aged 0.5 gestational days at noon on the day on which a vaginal plug was found.

Injection of antibody to pregnant mice

The anti-c-Kit monoclonal antibody, ACK2, which can block c-Kit function, has been described previously (Ogawa et al., 1991; Nishikawa et al., 1991; Yoshinaga et al., 1991). Pregnant mice were given purified ACK2 at a dose of 3 mg intravenously and 3 mg intradermally. Cell suspensions from the embryos were prepared as described (Ogawa et al., 1988) and analysed for the frequency of the in vitro colony forming cells. All experiments were repeated at least twice unless otherwise indicated, and similar observations were made in each separate experiment. In one experiment, the anti-CD4 monoclonal antibody, GK1.5 (Dialynas et al., 1983), and the anti-Mac-1 monoclonal antibody, M1/70 (Springer et al., 1979), were injected as class-matched control antibodies.

Microinjection of antibody to embryos

Embryos were microinjected by means of a simplified procedure according to the published method (Huszar et al., 1991). Pregnant mice were anesthetized with Nembutal (Abbott Laboratories, North Chicago, IL) at 50 mg/kg of body weight prior to laparotomy. The ACK2 solution was drawn into a glass microcapillary of about 50 μm diameter, which was attached to the automatic microdispenser Nanoject (Drummond Scientific Company, Broomall, PA). The uterus was held by forceps and the microcapillary was inserted into the decidual swelling. Each embryo was injected with a total of 0.44 μl solution containing 20 μg ACK2. Controls were injected with the same volume of saline.

Cell staining and flowcytometry

The cells were incubated on ice with an inactivated normal rabbit serum, then stained with the following monoclonal antibodies.

FITC-conjugated TER-119 (Ikuta et al., 1990) was used as an erythroid lineage marker. For staining c-Kit, biotin-labeled ACK4 (Ogawa et al., 1991) or, in some cases, the FITC-conjugated ACK2 was used. The stained cells were further incubated with streptavidin-PE (Becton Dickinson Immunocytometry Systems, San Jose, CA) and analyzed using an EPICS-Profile or an EPICS-Elite (Coulter Electronics Inc., Hialeah, FL). Cell sorting was performed by the EPICS-Elite.

In vitro colony assay

The cells were incubated in 1 ml of culture medium containing alpha-MEM (Gibco Laboratories, Grand Island, NY), 1.2% methylcellulose (Muromachi Kagaku Kogyo, Tokyo, Japan), 30% FCS (Whittaker Bioproducts, Walkersville, MD, Lot No.1M1137), 1% deionized BSA (Sigma Chemical Co., St. Louis, MO), 50 μM 2-mercaptoethanol (2-ME), antibiotics and 200 U/ml recombinant murine IL-3 (Hayashi et al., 1990) or 2 U/ml recombinant human Epo (Chugai Pharmaceutical Co. Ltd., Tokyo, Japan). Colony formation was monitored at 3 days (CFU-E) and 7 days (BFU-E, GM-CFC) after the inoculation (Iscove et al., 1974; Okada et al., 1991).

Cell culture

Fetal liver cells at various embryonic ages or adult bone marrow cells were passed through Sephadex G-10 (Pharmacia, Uppsala, Sweden) to eliminate stromal cells. Aliquots of the cells were analyzed for the initial frequency of colony forming cells reactive to IL-3 as described above. 500,000 of the remaining cells were suspended in 2 ml of RPMI1640 medium (Gibco) supplemented with 10% CS (Hyclone Laboratories, Logan, UT, Lot No.2151765), 50 μM 2-ME and antibiotics, then poured into a non-culture-grade dish 3.5 cm in diameter (Becton Dickinson Labware, Lincoln Park, NJ). 100 ng of murine SLF per ml was added to some dishes. After 9 days of incubation, the cells were harvested, counted and analyzed for the frequency of the colony forming cells. Murine SLF was produced by *Saccharomyces cerevisiae* and purified at the Chemo-sero-therapeutic Research Institute (Kumamoto, Japan).

The murine newborn calvaria-derived stromal cell line PA6 was maintained as previously described (Kodama et al., 1982; Sudo et al., 1989). 500,000 fetal liver cells were inoculated on a PA6 cell layer prepared in a T25 flask (Becton Dickinson Labware) and cultured for one week in the medium described above except for the inclusion of 5% CS. Cultured cells were harvested by gentle pipetting, passed through Sephadex G-10 to remove PA6 cells and tested in the colony assay.

RESULTS

Flow cytometric analysis of c-Kit and TER-119 expression in fetal hemopoietic organs

We first tested the expression of c-Kit in hemopoietic cells isolated from the fetal organs of various embryonic ages using the anti-c-Kit monoclonal antibody ACK4 and the erythrocyte lineage marker TER-119.

Most of the cells from the 8.5-day yolk sac expressed c-Kit but not TER-119 (Fig. 1A). The c-Kit expression ceased as the cells differentiated to TER-119$^+$ cells on the next day, whereas another c-Kit$^+$ TER-119$^-$ cell population appeared (Fig. 1A, Fig. 2). The c-Kit expression of this population was about five-fold higher than that of the c-Kit$^+$ cells that initially appeared in the yolk sac. The c-Kit^{hi} TER-119$^-$ cells increased to about 10% of the total hemopoietic cells of the yolk sac at 11.5 days of gestation and subsequently decreased (Fig. 1B). In contrast to the yolk sac, the c-Kit^{hi} TER-119$^-$ cells constituted the major population, that appeared first in the fetal liver (Fig. 1A, 11.5-day fetal liver). These c-Kit^{hi} cells did not express any other lineage markers including Mac-1 and B220 (data not shown). The proportion of this population rapidly decreased and the majority shifted to c-Kit$^-$ TER-119$^+$ cells (Fig. 1B). Both immature c-Kit$^+$ lineage marker$^-$ and mature c-Kit$^-$ lineage marker$^+$ cells were detected in the bone marrow at 16.5 days of gestation, when hemopoietic cells were first identified in the bone marrow (data not shown).

Expression of c-Kit in fetal hemopoietic progenitors

It has been established that hemopoietic progenitor cells clonable in vitro and in vivo are included in the c-Kit$^+$ cells of the adult bone marrow (Ogawa et al., 1991; Okada et al., 1991; Ikuta and Weissman, 1992). We next examined the correlation between the c-Kit expression and the clonogenic activity of the cells in fetal hemopoietic tissues.

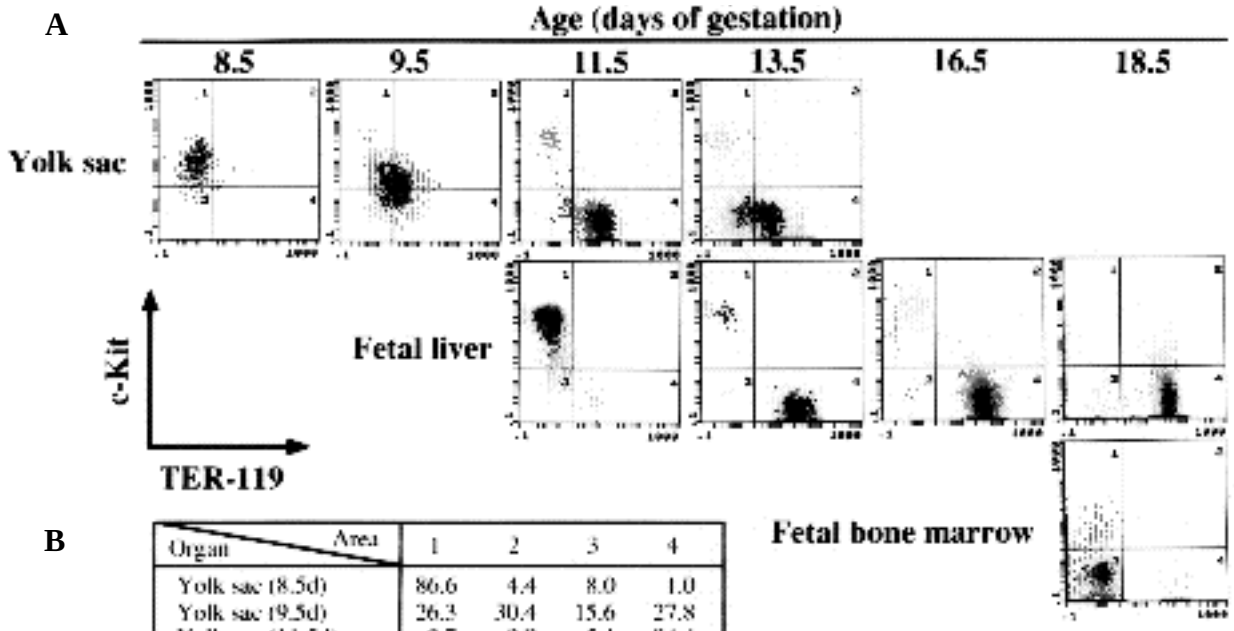
The c-Kit^{hi} TER-119$^-$ cells and c-Kit^{lo} TER-119^{lo} cells from the 9.5-day yolk sac or the c-Kit^{hi} TER-119$^-$ cells and c-Kit$^-$ TER-119$^+$ cells from the 12.5-day fetal liver were purified by fluorescence activated cell sorting (Fig. 2). The purified population was tested in an in vitro colony assay. Table 1 shows that most BFU-E and GM-CFC existed in the c-Kit^{hi} TER-119$^-$ fraction from both fetal organs. The c-Kit^{hi} fraction was also highly enriched with CFU-E in the 12.5-day fetal liver, although CFU-E was undetectable in the 9.5-day yolk sac. These results indicate that the hemopoietic progenitors in the fetal organs express c-Kit in a manner similar to that in the adult bone marrow.

Elimination of fetal hemopoietic progenitors by ACK2 injection

The results described above demonstrated the expression of c-Kit in fetal hemopoietic progenitors. However, this does not necessarily mean that c-Kit functions in these progenitors. Indeed, it has been reported that the proportions of GM-CFC and BFU-E are not affected in the fetal liver of the *W/W* mouse, which does not express functional c-Kit (Nocka et al., 1989). To determine whether or not the c-Kit is functionally required for the self-renewal of the hemopoietic progenitors of normal embryos, we attempted to block c-Kit function using the antagonistic anti-c-Kit monoclonal antibody ACK2. Our previous study showed that growth of hemopoietic progenitors was inhibited in the adult bone marrow by ACK2 injection (Ogawa et al., 1991). We also reported that ACK2 injected into pregnant mice is transferred into the embryos via the placenta (Nishikawa et al., 1991).

First, normal adult female mice were injected intraperitoneally with 2.5 mg purified ACK2 and the contents of CFU-E and GM-CFC in the bone marrow were examined 2 days later. Consistent with our previous report, the number of CFU-E and GM-CFC decreased markedly while the total number of bone marrow cells remained unaffected (Table 2).

We next injected 6 mg ACK2 (3 mg intravenously and 3 mg intradermally) into the pregnant mice 12.5-15.5 days



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Organ \ Area	1	2	3	4
Yolk sac (8.5d)	86.6	4.4	8.0	1.0
Yolk sac (9.5d)	26.3	30.4	15.6	27.8
Yolk sac (11.5d)	9.7	0.9	5.1	84.4
Yolk sac (13.5d)	4.1	1.3	27.5	67.0
Liver (11.5d)	84.1	2.8	6.8	6.3
Liver (13.5d)	17.8	2.7	6.8	72.7
Liver (16.5d)	6.2	4.3	3.5	86.0
Liver (18.5d)	4.8	4.0	8.3	82.9
Bone marrow (18.5d)	14.8	0.7	74.6	9.9

Fig. 1. Expression of c-Kit and the erythroid lineage marker TER-119 on cells disaggregated from mouse hemopoietic tissues at various embryonic ages. Cells were incubated with normal rabbit serum, then biotin-labeled anti-c-Kit (ACK4) and FITC-conjugated TER-119 antibodies were added. Cells were further stained with streptavidin-PE, and analysed by flowcytometry. (A) Dot-plotted two-color profile. The ordinate and abscissa represent log fluorescence intensity. The vertical and horizontal lines indicate the threshold of fluorescence intensity of negative control staining. (B) The percentage of cells that appeared in each quartered area.

postcoitum and counted the number of CFU-E and GM-CFC in the bone marrow and liver of the fetuses 2 days after the injection. Contrary to the previous report on the mutant mouse, a remarkable reduction of GM-CFC as well as CFU-E was observed in the ACK2-treated embryos (Table 2). Lineage-committed myeloid progenitors responding to granulocyte/macrophage colony stimulating factor (GM-CSF) or CSF-1 were also eliminated from the fetal liver (data not shown). Because the addition of ACK2 did not affect the formation of erythroid and myeloid colonies from fetal liver cells in semisolid medium, the decrease of the progenitors was not due to the effect of ACK2 carried into the assay culture (data not shown). These results indicate that not only the erythroid, but also the myeloid progenitors at various differentiation stages depend on c-Kit for maintenance in the fetal organs from at least 12.5 days of gestation. When the mouse was given the same dose of ACK2 at 12.5 days of gestation and the colony assay was delayed until 17.5 days of gestation, the total cellularity and the number of the hemopoietic progenitors remained reduced in the fetal liver (Table 2). The production of blood cells in the fetal bone marrow was also affected in the embryos despite the fact that hemopoiesis in the bone marrow started long after the injection of ACK2. A simi-

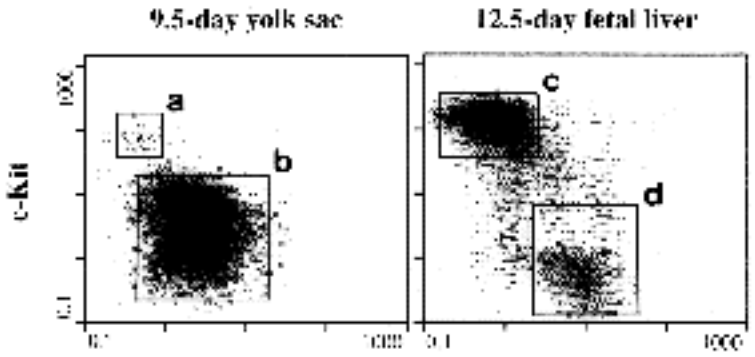

Fig. 2. Cell fractionation by fluorescence activated cell sorting. The cells disaggregated from the 9.5-day yolk sac and the 12.5-day fetal liver were stained with anti-c-Kit (ACK4) and TER-119 antibodies, then fractionated by the EPICS-Elite cell sorter. Sorting gates are indicated by the boxes (a-d, corresponding to Table 1).

Table 1. Colony formation by sorted yolk sac and fetal liver cells

Organ	Cell	No. of CFC/10^5 cells*		
		CFU-E	BFU-E	GM-CFC
9.5-day yolk sac	unfractionated	ND§	53±25	199±29
	c-Kit^{hi} TER-119$^-$ (a)	ND	350±300	3,500±1,120
	c-Kit^{lo} TER-119^{lo} (b)	ND	9±4	32±11
12.5-day fetal liver	unfractionated	9,340±1,060	101±14	759±107
	c-Kit^{hi} TER-119$^-$ (c)	20,300±1,710	164±38	1,720±147
	c-Kit$^-$ TER-119$^+$ (d)	164±36	3±6	16±19

*Cells were fractionated as described in the legend to Fig. 2, and the frequency of colony forming cells was determined. Values of colony forming cells are mean±s.d. of triplicate cultures.
§Not detected.

lar long-lasting depletion of the hemopoietic progenitors was observed in the fetuses even when the dose of ACK2 was reduced to 2 mg (Table 3 and data not shown). Nevertheless, for the reasons described later, we used a threefold saturating dose of ACK2 throughout these experiments.

To eliminate the possibility that the reduction of hemopoietic progenitor cells is due to a nonspecific effect of the rat antibody, we treated the pregnant mice with the anti-CD4 monoclonal antibody, GK1.5, and the anti-Mac-1 monoclonal antibody, M1/70, as class-matched control antibodies. The numbers of total cells and colony forming cells in the fetal livers were not affected by the treatment of these antibodies, indicating that the blockade of fetal hemopoiesis by ACK2 is not a nonspecific effect of the rat monoclonal antibody (Table 3).

Effect of ACK2 on the early phase of fetal liver hemopoiesis

We next attempted to clarify whether the c-Kit and its ligand are required in earlier phase of fetal liver hemopoiesis. Pregnant mice were injected with ACK2 at 10.5 or 11.5 days postcoitum as described above, and the number of hemopoietic progenitors in the fetal liver was examined 2 days later. In the 13.5-day fetal liver, the number of CFU-E and GM-CFC significantly decreased compared with the control mouse (Table 4). On the other hand, the numbers of GM-CFC were less affected in the 12.5-day fetal liver, although CFU-E were affected significantly. Nevertheless, injection of ACK2 at 10.5 days of gestation reduced the number of GM-CFC in the 13.5-day fetal liver. These results suggested that ACK2 could not inhibit the growth of myeloid progenitors in the fetal liver before 12.5 days of gestation. To confirm this, ACK2 was injected into pregnant mice at 11.5 or 12.5 days postcoitum and the number of progenitors was examined at 24 hours later. ACK2 exposure for 24 hours was sufficient to reduce the number of CFU-E and GM-CFC when given at 12.5 days of gestation, whereas the same treatment was less effective at 11.5 days of gestation (Table 4).

To exclude the possibility that even an excess of ACK2 injected maternally cannot reach the embryos before 12.5 days of gestation, we isolated 12.5-day fetal liver cells from mice given ACK2 24 hours previously, then stained them with both FITC-conjugated ACK2 and biotin-labeled ACK4. As shown in the staining profile of control embryos in Fig. 3A, these two antibodies recognize different determinants on the c-Kit molecule and do not interfere with c-Kit binding each other. On the other hand, the fetal liver cells isolated from the ACK2-treated mouse were positively stained with ACK4 but not with ACK2, indicating that the determinant on the c-Kit molecule was saturated with ACK2 which was transported via the placenta (Fig. 3B).

Effect of ACK2 on hemopoietic progenitors in the yolk sac

The present and previous studies showed c-Kit expression on blood cells in the early yolk sac (Orr-Urtreger et al., 1990; Palacios and Nishikawa, 1992). It was also reported that the c-Kit ligand is weakly expressed in the yolk sac (Matsui et al., 1990). These suggest a role for c-Kit and its

Table 2. Effect of ACK2 injection on fetal hemopoietic progenitor cells

Days of gestation			Percent of control value (No. of cells/organ)*		
Injection	Assay	Organ	Total cells	CFU-E	GM-CFC
–	–	adult bone marrow§¶	92.3 (1.2×10^7)	2.0 (729±425)	17.5 (8,080±1,940)
15.5	17.5	fetal bone marrow§	55.0 (8.8×10^3)	2.7 (0.4±0.8)	10.8 (10±2)
14.5	16.5	fetal liver	35.7 (1.5×10^7)	4.8 (4,880±4,880)	11.9 (4,960±1,850)
12.5	14.5	fetal liver	100.0 (4.4×10^6)	10.1 (11,100±2,770)	3.4 (599±133)
12.5	17.5	fetal liver	9.6 (2.5×10^6)	0.6 (679±309)	0.1 (37±74)
12.5	17.5	fetal bone marrow§	18.5 (2.4×10^3)	<1.4 (<0.2)	<0.4 (<0.2)

*6 mg ACK2 per animal was injected into pregnant mice. The number of total cells and colony forming cells per organ was determined at 2 or 5 days after the injection. Values of colony forming cells are mean±s.d. of triplicate cultures.
§Number of cells per femur is indicated.
¶Adult mouse was injected with 2.5 mg ACK2, then colony assay was performed 2 days thereafter.

Table 3. Effect of antibody injection on hemopoietic progenitor cells in fetal liver

	No. of cells/fetal liver*		
Antibody	Total cells ($\times 10^7$)	CFU-E ($\times 10^5$)	GM-CFC ($\times 10^4$)
PBS§	5.53	3.66±0.23	4.33±0.36
ACK2	1.07	0.17±0.03	0.70±0.01
GK1.5	5.67	3.74±0.27	3.48±0.34
M1/70	5.93	3.83±0.28	4.52±0.38

*2 mg antibody per animal was injected intravenously into the pregnant mice at 14.5 days postcoitum. The number of total cells and colony forming cells per fetal liver were determined at 2 days after the injection. Values of colony forming cells are mean±s.d. of triplicate cultures.
§As a control, 0.5 ml of PBS was injected.

ligand in yolk sac hemopoiesis. However, in contrast with the significant deleterious effects of mutant alleles of *W* and *Sl* loci on the fetal liver erythropoiesis, the number of yolk sac-derived nucleated erythrocytes was less affected in the *W/W* embryo and it was almost normal in *Sl/Sl*^{d} embryo (Russell et al., 1968; Chui and Russell, 1974; Chui and Loyer, 1975). This discrepancy prompted us to examine the effect of ACK2 on the hemopoietic progenitors in the yolk sac. Because the placenta does not functionally mature until 10 days of gestation, ACK2 was microinjected into embryos in utero before 10.5 days of gestation (see Materials and Methods). Saturation of ACK2 was confirmed as described above (Fig. 3C,D).

ACK2 injection reduced the number of CFU-E to about half the control level in the 10.5-day yolk sac, whereas we detected only small numbers of CFU-E in the 9.5-day yolk sac even in the control embryos (Table 5). Of interest is that the number of BFU-E and GM-CFC in the yolk sac increased rather than decreased after ACK2 injection. When ACK2 was injected at 12.5 days of gestation, the number of BFU-E rapidly decreased in the fetal liver (Table 5) as in the bone marrow of the ACK2-treated adult mouse (data not shown). The counts of GM-CFC included myeloid cell colonies and mixed type colonies containing myeloid and erythroid lineage cells. Both types of colonies were reduced equally in the 13.5-day fetal liver (data not shown).

In vitro proliferation of GM-CFC in response to c-Kit ligand

Our present results suggest that GM-CFC can be maintained without the c-Kit function in vivo before 12.5 days of gestation. We next tested the growth ability of myeloid progenitors in vitro in response to the c-Kit ligand.

Adult bone marrow cells were maintained in suspension culture in the presence of SLF for 9 days as a control. The total number of cells increased 9-fold during the culture period (Table 6). May-Giemsa staining showed that half of the cells were mature neutrophils and the remainder consisted of promyelocytes and blasts (data not shown). GM-CFC responding to IL-3 increased 17-fold in the presence of SLF alone.

Fetal liver cells of various embryonic ages were cultured under the same conditions. The total number of cells increased 3 to 10-fold, although mast cells dominated (65% in the cultured 13.5-day fetal liver cells). In contrast to the bone marrow cells, GM-CFC derived from fetal livers could not be maintained with SLF alone (Table 6). Previous reports indicated that IL-6 enhanced the growth promoting activity of SLF for bone marrow hemopoietic progenitors (Tsuji et al., 1991; Bodine et al., 1992). However, fetal liver-derived GM-CFC did not exceed the initial number even in the presence of SLF and IL-6 (data not shown).

Myeloid cells as well as B lymphocytes can be propagated from fetal liver in culture with a stromal cell clone (Ogawa et al., 1988). The stromal cell clone PA6 produces all of the molecules required for in vitro proliferation of the hemopoietic progenitor cells, including SLF but neither IL-3 nor GM-CSF. Finally we cultured the 11.5 to 13.5-day fetal liver cells on a cell layer of the PA6 clone for a week. The total number of cells increased in each culture four- to six-fold (Table 6). These included mature neutrophils, macrophages and mast cells (44, 32 and 4% in the cultured 12.5-day fetal liver cells). In the cultured 13.5-day fetal liver cells, GM-CFC responding to IL-3 increased about 3-fold on the PA6 cell layer, whereas GM-CFC could not be maintained in cultured 11.5- and 12.5-day fetal liver cells. The inability of GM-CFC to proliferate may not be due to the existence of some inhibitory factors secreted by 12.5-day fetal liver cells, since GM-CFC increased when 13.5- and 12.5-day fetal liver cells were co-cultured.

These results suggested that GM-CFC in the 13.5-day fetal liver can proliferate in response to some growth signals expressed by PA6, which may include SLF, whereas these putative molecules cannot support self-renewal of GM-CFC in the fetal liver at earlier embryonic ages.

DISCUSSION

It is established that c-Kit is expressed in the hemopoietic tissues of the mouse embryo (Orr-Urtreger et al., 1990;

Table 4. Effect of ACK2 injection on hemopoietic progenitor cells in fetal liver

Days of gestation		Percent of control value (No. of cells/fetal liver)*		
Injection	Assay	Total cells	CFU-E	GM-CFC
11.5	13.5	46.2 (1.3×10^6)	15.3 (7,800±1,770)	13.8 (1,060±198)
10.5	12.5	67.7 (2.1×10^5)	37.0 (8,280±1,470)	67.1 (735±11)
10.5	13.5	42.9 (1.2×10^6)	48.7 (24,800±2,700)	23.9 (1,830±351)
11.5	12.5	103.2 (6.4×10^5)	30.4 (8,830±1,180)	68.6 (1,180±284)
12.5	13.5	124.0 (3.1×10^6)	12.6 (10,500±3,140)	15.5 (1,520±239)

*6 mg ACK2 per animal was injected into the pregnant mice. The numbers of total cells and colony forming cells per fetal liver were determined at 1 or 3 days after the injection. Values of colony forming cells are mean±s.d. of triplicate cultures.

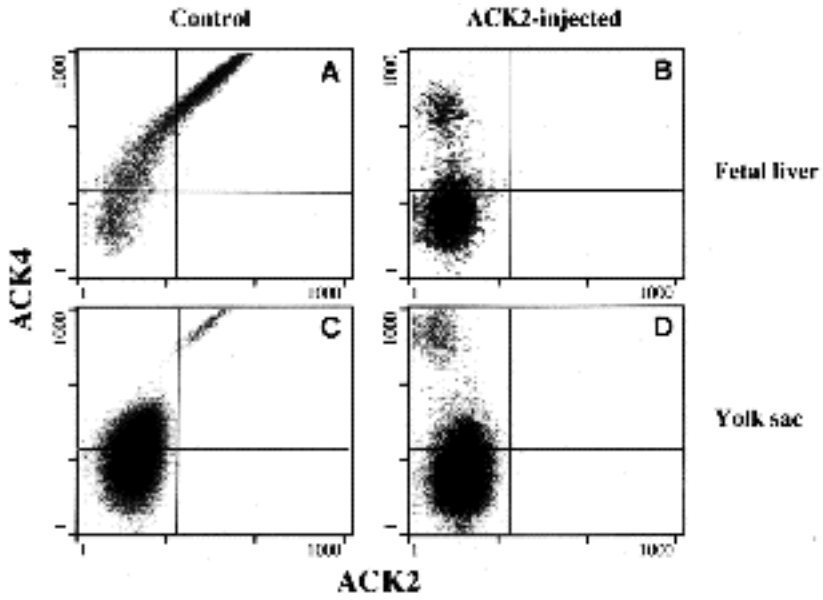

Fig. 3. Saturation of the cell surface with ACK2 administered to the fetuses. 12.5-day fetal liver cells and 9.5-day yolk sac cells of control (A,C) and ACK2-treated (B,D) embryos were stained with ACK2 (FITC) and ACK4 (PE), which recognize different determinants on c-Kit.

Motro et al., 1991; Ikuta and Weissman, 1992; Palacios and Nishikawa, 1992). Present study using flowcytometry did not merely corroborate these previous reports but provided two more features of c-Kit expression in embryonic hemopoietic cells. First, the hemopoiesis in the yolk sac and the fetal liver starts from cells that express c-Kit. These cells give rise to c-Kit$^-$ lineage marker$^+$ mature cells and become a minor population in each tissue within two days of the initiation of hemopoiesis. Secondly, most hemopoietic progenitor cells in the embryo express high levels of c-Kit, as do the progenitors in the adult bone marrow. The ability of progenitors to generate mature erythrocytes in a short period of time would contribute to coordinative recruitment of erythrocytes into the generating systemic circulation.

The next question addressed was whether the c-Kit expressed on the surface of the hemopoietic progenitors is functional. We administered ACK2, an anti-c-Kit monoclonal antibody that is antagonistic to c-Kit, into embryos to determine whether it would block hemopoiesis in embryonic tissues. CFU-E, BFU-E and GM-CFC were rapidly eliminated from the fetal liver when ACK2 was injected after 12.5 days of gestation. This elimination was not due to a cytotoxic effect of ACK2 since more than half of the c-Kit$^+$ cells remained in the liver of ACK2-treated embryos. Thus, these results indicated that c-Kit function is essential for the proliferation of both the erythroid and myeloid progenitors in the fetal liver after 12.5 days of gestation. However, our conclusion is in discord with the phenotype of the *W/W* mutant embryos in which the proportion of CFU-E in fetal liver is reduced whereas BFU-E and GM-CFC are not affected (Nocka et al., 1989). The proportion of CFU-E but not BFU-E was reduced in the fetal liver of *Sl/Sl*^{d} embryos (Chui et al., 1978). Moreover, CFU-S can be generated in the complete absence of SLF in *Sl/Sl* embryos (Ikuta and Weissman, 1992). One explanation for this discrepancy is that some other molecules compensate for the lack of SLF/c-Kit function in the mutant mice. The abrupt blockade of c-Kit function by an antagonistic antibody reduces the hemopoietic progenitor cells in the normal mouse, which depend upon SLF/c-Kit for their self-renewal. In contrast, progenitors in the mutant mice, which congenitally lack the function of SLF/c-Kit, can be adapted to some other signaling molecules during development. This may reflect the existence of multiple extracellular signal molecules that can regulate the proliferation and differentiation of hemopoietic cells. However, IL-3 and GM-CSF that have growth promoting activity for hemopoietic stem cells are unlikely to be such compensatory molecules since they are not expressed in the fetal liver (Azoulay et al., 1987; Dallman et al., 1991).

Of interest is that ACK2 injected on 12.5 days of gestation suppressed the generation of blood cells in the fetal

Table 5. Effect of ACK2 injection on fetal hemopoietic progenitor cells

	Percent of control value (No. of cells/organ)*			
Organ	Total cells	CFU-E	BFU-E	GM-CFC
9.5 d yolk sac	103.4 (6.1×10^4)	127.3 (4±1)	128.9 (47±13)	156.0 (473±42)
10.5 d yolk sac	147.6 (6.2×10^4)	49.9 (98±22)	148.1 (59±16)	140.9 (711±27)
12.0 d fetal liver	83.3 (1.5×10^5)	65.8 (12,600±2,000)	114.8 (70±16)	88.4 (1,140±116)
13.5 d fetal liver	94.4 (1.7×10^6)	26.2 (13,000±1,910)	30.7 (229±102)	29.9 (1,980±260)

*The numbers of total cells and colony forming cells in the yolk sac and fetal liver of the mice injected with ACK2 before 24 hours were determined. Values of colony forming cells are mean±s.d. of triplicate cultures.

Table 6. Growth ability of GM-CFC from adult bone marrow and fetal liver

Cultured cells	Stimuli	Total cell recovery /culture (×10^6)	No. of GM-CFC/culture* Input	Recovery
Adult bone marrow	SLF**	4.6	2,740±137	46,500±2,650
12.5 d fetal liver	SLF	5.7	2,100±155	285±285
13.5 d fetal liver	SLF	2.7	1,200±65	362±281
14.5 d fetal liver	SLF	2.0	1,440±78	585±254
15.5 d fetal liver	SLF	1.7	1,220±128	643±125
Adult bone marrow	PA6§	3.3	770±43	4,510±93
11.5 d fetal liver	PA6	2.0	650¶	31±14
12.5 d fetal liver	PA6	3.2	1,560±269	872±272
13.5 d fetal liver	PA6	3.1	875±21	2,970±66
12.5 d fetal liver+ 13.5 d fetal liver	PA6	3.4	1,220±135	1,850±24

*Values of GM-CFC are mean±s.d. of triplicate determinations and representative of more than two independent experiments.

**5×10^5 cells were cultured with recombinant SLF for 9 days.

§5×10^5 cells were cultured on the monolayer of PA6 stromal cell line for 7 days.

¶Data from one dish.

bone marrow. The offspring from mice given a single shot of ACK2 on 12.5 days of gestation die perinatally of aplastic anemia (Nishikawa et al., 1991). These results demonstrate that the hemopoietic stem cells in the fetal liver proliferate actively after about 12 days of gestation in response to SLF, and this process is essential for the generation of the stem cell pool sufficient for initiating marrow hemopoiesis.

In contrast to the remarkable effects of ACK2 on the fetal liver after 12.5 days of gestation, the number of BFU-E and GM-CFC did not decrease in the yolk sac and fetal liver of embryos given ACK2 before 12.5 days of gestation. CFU-E was also less affected in the early hemopoietic organs. These results suggested that c-Kit is not required for the proliferation of the hemopoietic progenitors before 12.5 days of gestation. Thus, we propose that hemopoiesis of the mouse embryo can be divided into two phases, one is the c-Kit-independent fetal type hemopoiesis and the other is the c-Kit-dependent adult type hemopoiesis. The transition from the fetal to the adult type occurs at 12-13 days of gestation.

Of importance is the fact that the fetal type hemopoiesis differs from the adult type hemopoiesis in growth requirements. Whether this difference reflects properties of the hemopoietic stem cell itself or that of the microenvironment remains to be elucidated. Earlier studies have shown that hemopoietic stem cells in the yolk sac and fetal liver before 12 days of gestation cannot reconstitute the adult hemopoietic system of the *W/W*^{v} mouse (Harrison et al., 1979; Sonoda et al., 1983). In contrast, adult bone marrow cells and 13- to 15-day fetal liver cells can seed the liver of early fetuses and reconstitute the hemopoiesis of the *W* mutant mouse, indicating that the embryonic microenvironment is sufficient for adult type hemopoietic stem cells (Fleischman and Mintz, 1979, 1984). These results suggest that the hemopoietic cell autonomous property differs between fetal and adult type hemopoiesis. It is of interest to note in this context that the proliferative response of fetal hemopoietic cells to recombinant soluble SLF remains poor even after becoming the c-Kit-dependent adult type. Since c-Kit is expressed in the hemopoietic stem cells throughout embryonic life, the molecules that determine the SLF reactivity would lie downstream of the c-Kit receptor. Mucenski et al. (1991) reported that disruption of the c-*myb* gene by homologous recombination suppressed the later wave of fetal hemopoiesis starting at 13 days of gestation, while earlier erythropoiesis occurred normally in this particular mouse. Thus, it would be useful to know whether c-Myb is present downstream of the c-Kit signaling pathway.

Finally, if c-Kit as well as c-Myb is not functionally required for early fetal hemopoiesis, what kind of signaling pathway could regulate instead? The c-Kit molecule is a receptor tyrosine kinase which belongs to the platelet derived growth factor (PDGF) receptor/CSF-1 receptor subfamily. Recently, genes encoding other murine receptor tyrosine kinases, such as *flk-1* and *flk-2/flt-3*, which come under this category, have been cloned (Matthews et al., 1991a,b; Rosnet et al., 1991). Flk-1 is highly homologous to human Flt-1, a receptor for vascular endothelial growth factor (Shibuya et al., 1990; de Vries et al., 1992). The receptor tyrosine kinases that belong to the PDGFR/CSF-1R subfamily seem to participate in regulation of proliferation of cells originating from the mesenchyme as well as germ cells and those of neural crest-origin. Thus it is likely that some molecules in this family take part in c-Kit-independent early hemopoiesis. Indeed, *flk-2/flt-3* is expressed in fetal hemopoietic stem cells. The roles of these receptors in fetal hemopoiesis, if any, must be elucidated.

In conclusion, we identified two successive phases of fetal hemopoiesis, one is independent of c-Kit and the other is dependent upon c-Kit. The transition from the former to the latter occurs after the shift of the hemopoietic site from the yolk sac to the fetal liver. Thus, even if the hemopoieses in the yolk sac and the fetal liver originate from separate lineages of stem cell, these two successive phases do not simply reflect different properties of these lineages. Nevertheless, expression of the c-*kit* gene may be a useful marker to identify hemopoietic progenitors in early embryonic organs and to trace their origins.

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