Recruited peripheral blood monocytes participate in the liver extramedullary hematopoietic milieu*

Ayako Nakamura-Ishizu, Shunichi Morikawa, Kazuhiko Shimizu, and Taichi Ezaki

Department of Anatomy and Developmental Biology, Tokyo Women's Medical University, Tokyo, Japan

Summary. The hematopoietic microenvironment has been investigated and well defined in the bone marrow. However, there is a lack of studies on the extramedullary hematopoietic milieu such as in the liver, to which hematopoietic stem cells migrate and there commence hematopoiesis under pathological conditions such as bone marrow failure. We induced extramedullary hematopoiesis by phenylhydrazine in the adult mouse liver and investigated the immunohistochemical, ultrastructural, and molecular changes within this organ. Using an intravital lectin injection technique, we found numerous monocytes attached to the central vein prior to hematopoietic foci formation. These cells were later incorporated into the hematopoietic foci. An increase in the mRNA expressions of the monocyte attracting chemokine CCL-2 (MCP-1) was noted in the central vein region as well as in cells within the hematopoietic foci. Together with local liver components, we regard these monocytes as components of the extramedullary hematopoietic milieu. We conclude that the recruitment of extra-hepatic monocytes is an important event during extramedullary hematopoiesis in the liver and that these monocytes participate in the liver hematopoietic microenvironment.

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Address for correspondence: Prof. Taichi Ezaki, MD, PhD, Department of Anatomy and Developmental Biology, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo, 162-8666, Japan
Tel & Fax: +81-3-5269-7407
Email: ezakit@research.twmu.ac.jp

Introduction

The bone marrow is the major hematopoietic organ maintaining homeostasis of mature hematopoietic cells during adulthood. A specialized microenvironment supports the development and maturation of hematopoietic cells (Schofield, 1978). In the bone marrow, osteoblasts (Taichman, 2005), macrophages (Hanspal and Hanspal, 1994), sinusoidal endothelial cells (Kopp *et al.*, 2005), reticular cells (Penn *et al.*, 1993), fibroblasts (Zuckerman *et al.*, 1985) and adipocytes (Yin and Li, 2006) comprise as the hematopoietic milieu. Organs other than the bone marrow are known to participate in hematopoiesis. Hematopoietic stem cells (HSCs) enter the circulation from the bone marrow at certain rates homeostatically (Wright *et al.*, 2001) and can lodge in the embryonic hematopoietic tissues such as the liver and spleen for hematopoiesis during bone marrow failure (O'Keane *et al.*, 1989), severe anemia (Taher *et al.*, 2006), or myeloproliferative diseases (Ahmed and Chang, 2006). However, the hematopoietic microenvironment in these extramedullary sites remains poorly defined.

Macrophages are known as a crucial component of the hematopoietic microenvironment (Weiss, 1976). A central macrophage surrounded by erythroid cells composes erythroblastic islands as a functional unit for erythroid hematopoiesis (Chasis, 2006). The phenotype and functions of the central macrophage in the erythroblastic islands have been well investigated in the bone marrow (Lee *et al.*, 1988). However, the origin and phenotype of the central macrophages during liver extramedullary hematopoiesis is unclear. Some reports have speculated that Kupffer cells in the liver undergo phenotypic changes to nurse hematopoietic cells during extramedullary hematopoiesis (de Jong *et al.*, 1994; Klein *et al.*, 2007). Identification of the origin and signals that recruit and activate these macrophages is necessary to further

understand hematopoietic stem cell biology.

We here show that, during extramedullary hematopoiesis in the adult mouse liver, massive numbers of peripheral monocytes infiltrate into the liver. Our evidence clearly indicates that the origin of some macrophages in the erythroblastic islands formed in the adult liver is extra-hepatic. These recruited monocytes then participate in the extramedullary hematopoietic milieu in the liver.

Materials and Methods

Animal

Male BALB/c mice, 8–10 weeks of age, were purchased from Japan SLC Inc. (Shizuoka). They were maintained in air filtered clean rooms and fed with sterilized standard laboratory chow and water at the Institute of Laboratory Animals, Tokyo Women's Medical University. The following experiments were conducted in accordance with the regulations of the Institute of Laboratory Animals for Animal Experimentation at Tokyo Women's Medical University. On use, all mice were anesthetized by an intramuscular injection of a mixture of ketamine (87 mg/kg body weight) and xylazine (13 mg/kg body weight).

Induction of extramedullary hematopoiesis in the liver by phenylhydrazine (PHZ) in splenectomized mice

Extramedullary hematopoiesis in the liver was induced with phenylhydrazine hydrochloride (PHZ: Sigma-Aldrich, St. Louis, MO, USA) according to the method proposed by Adler *S et al.* (Adler and Trobaugh, 1978) with some modifications (Fig.1). Briefly, mice were splenectomized 7–10 days before PHZ injection. Subcutaneous injection of 1.2 mg PHZ in 0.1 ml phosphate buffered saline solution (PBS: Fuso Chemicals, Osaka) was performed for three consecutive days. Control animals were splenectomized animals injected with 0.1 ml of buffered saline subcutaneously. The first injection day was denoted as day 0. Animals were dissected on days 2, 4, 7, 14 and 21.

Intravital lectin staining and perfusion fixation

In order to delineate blood vessel endothelial cells and monocytes, an intravital lectin injection was performed prior to perfusion fixation of the animals. FITC-conjugated *Ricinus communis agglutinin-I* (RCA-I) (200 μ l; Vector Laboratories Inc., Burlingame, USA),

diluted in PBS at a concentration of 0.5 mg/ml, was injected individually into the tail vein of the mice. Ten minutes after injection, the animals were fixed by perfusion of 4% paraformaldehyde (PFA) in PBS through the left ventricle for 5–10 min at pressures ranging from 120 to 80 mmHg. Their livers were removed and post-fixed in 4% PFA in PBS at room temperature (RT) for 1 h. After washing with PBS and immersion in 30% sucrose in PBS, the organs were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, USA) and snap frozen in liquid nitrogen for cryosection.

Immunohistochemistry

Cryosections (16 μ m thick) of the organs were immersed in a 1% Block-ace blocking solution (Dainippon Seiyaku Ltd., Tokyo) at RT for 15 min. The sections were then treated with primary antibodies to Type IV collagen (LSL CO., Tokyo), LYVE-1 (AngioBio, CA, USA) or CD45 (PharMingen, NJ, USA) and corresponding fluorescent conjugated secondary antibodies (Jackson ImmunoResearch, PA, USA). Thereafter, the sections were fixed with 4% PFA for 10 min and mounted in Vectashield (Vector Laboratories Inc.). The fluorescent images were observed under a Leica TCS-SL confocal laser scanning microscope (Leica, Wetzlar, Germany). Concomitantly, hematoxy-eosin stained samples were made and observed under an Olympus BX-51 light microscope (Olympus, Tokyo).

For bright field immunohistochemistry, sections were reacted with either F4/80 (Serotec, Kidlington, UK) or desmin (Abcam, Cambridge, UK) antibodies, followed by their corresponding peroxidase conjugated secondary antibodies. A peroxidase reaction was developed at RT for 10 min in a reaction mixture of 10 mg of 3'-diaminobenzidine hydrochloride (DAB: Dojin Chemicals, Kumamoto) and 10 μ l of 30% H₂O₂ in 50 ml of PBS. The sections were counterstained with hematoxylin.

Fig. 1. Protocol of extramedullary hematopoiesis.

PKH26 labeling and transplantation of peripheral blood monocytes

In order to determine the origin of the recruited cells that appeared during extramedullary hematopoiesis, we designed a cell transfer experiment with various combinations of donor cells and host mice (Fig. 2). We first labeled phagocytic cells in the peripheral blood from either control (splenectomized mice without PHZ treatment) or day 4 mice and transplanted them individually into recipient mice either under or not under the same hemolytic stress. Donor mice were anesthetized and opened through the abdomen. Whole

blood samples were obtained from the inferior vena cava using a 23G needle. A PKH26 red fluorescent cell linker kit (Sigma-Aldrich Co.) was used for monocyte labeling according to the manufacturer's manual. Labeled cells were resuspended in calcium and magnesium-free PBS at a concentration of 4×10^8 cells/ml and 100 μ l of this solution was transfused into the tail vein of the recipient mice. Livers of the perfusion fixed mice were obtained 4 h and 3 days (equivalent to day 7 of extramedullary hematopoiesis protocol) after the transplantation. For 4-h samples, the number of PKH26 positive cells per field at a magnification of 200 times was counted. For 3-day samples, the ratios of hematopoietic foci that contained at least one PKH26 positive cell to the total focus numbers were calculated for 100 foci per sample.

Fig. 2. Transplantation protocol of PKH26 labeled monocytes.

Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) for chemokines CCL-2 and CCL-5

Splenectomized mice treated with PHZ were sacrificed by cervical dislocation on days 2, 4 and 7. Total RNA in the liver was extracted using ISOGEN (Nippon Gene Co., Inc., Japan). SuperscriptTM III reverse transcriptase (Invitrogen, USA) was used for the synthesis of cDNA. Primers of the chemokines are shown in Table 1. Conditions for DNA amplifications were set as follows: 26 cycles of DNA denaturation at 94°C for 1 min, an annealing step at 56°C for 1 min, and strand extension at 72°C for 45 sec. PCR amplification of α -actin and its band intensity were used to determine the quality and quantity of the cDNA of the samples for semi-quantitative analyses.

Table 1. PCR Primers for chemokines and their receptors

	Primer sequence (forward)	Primer sequence (reverse)
Chemokine		
CCL-2	AGCACCAGCCAACTCTCACT	TCTGGACCCATTCCTTCTTG
CCL-5	CCCTCACCATCATCCTCACT	GGGATTACTGGAGTGGCATC
CX3CL-1	GTTGGCTCCTGAGAGTGAGG	CAAAATGGCACAGACATTGG
Chemokine receptor		
CCR-1	GTTGGGACCTTGAACCTTGA	CTAGGACATTGCCCACCACT
CCR-2	CCTGCAAAGACCAGAAGAGG	GATGGCCAAGTTGAGCAGAT
CCR-3	TTCTACCGGCCCTCACATAC	CTGGGAACGTGTTGTTGATG
CCR-5	ATTCTCCACACCCTGTTTCG	CGTTTGACCATGTGTTTTCG
CX3CR-1	CCTGCCTCTGAGAAATGGAG	ATCTCTCCAGCCCCTGAAAT

In situ hybridization of CCL-2 and bright field immunohistochemistry of adjacent sections

In situ hybridization (Lindeberg and Johansen, 1990) was conducted in order to determine sites of chemokine production. Complementary DNA probes were prepared through RT-PCR amplification using the primers as described previously in the protocols. PCR products were labeled with digoxigenin using a DIG high prime (Roche Inc., USA).

In situ hybridization was conducted using a DIG high prime detection starter kit (Roche Diagnostics Corp., USA) with modifications. Paraffin sections (3 μ m) of day 4, day 7 and control livers were dewaxed and then immersed in 3% hydrogen peroxide in a methanol solution for 30 min to inactivate endogenous peroxidase. They were then placed in 0.2N HCl for 10 min, 2X SSC (300 mM sodium chloride, 30 mM sodium citrate, pH 7.0) at 70°C for 30 min, and then were treated with proteinase K (Nippon Gene) at 37°C for 1 h. The sections were placed in an acetylation solution at RT for 10 min. Hybridization probes were denatured and hybridization was conducted at 42°C overnight. A sheep anti-DIG antibody (Roche Diagnostics, USA) was used at a dilution of 1:500 at RT for 30 min. Sections were rinsed and treated with peroxidase-conjugated donkey anti-sheep IgG (Jackson ImmunoResearch, USA) at RT for 1 h. Peroxidase reaction was developed in a DAB reaction mixture. Serial paraffin sections were made for bright field immunohistochemistry.

Statistical analyses

Statistical analyses were conducted using the student *t*-test. P-values below 0.01 were regarded as statistically significant.

Results

Induction of extramedullary hematopoiesis in the liver

Extramedullary hematopoiesis was induced in the liver of the PHZ-treated splenectomized mice. No obvious sign of hematopoiesis was seen on day 4 (Fig. 3a). Numerous hematopoietic foci were identified chiefly distributed in the periportal regions by day 7 (Fig. 3b arrows). Electron microscopic analyses of the liver showed numerous hematopoietic foci, mostly consisting of erythroid cells at day 7 (Fig. 3c). A thin layer of endothelial cells surrounded most of the hematopoietic foci, indicating that

the hematopoietic foci formed primarily intra-luminally within the expanded sinusoids (Fig. 3c arrowheads).

Involvement of the RES cells during extramedullary hematopoiesis

We used an intravital injection of fluorescent conjugated lectin (RCA-I) to delineate the sinusoidal endothelia and phagocytic cells of the liver according to our previous report (Nakamura-Ishizu *et al.*, 2008). On day 4, multiple round cells, which exhibited a granular lectin staining pattern, were observed attached to the endothelia of central veins and sinusoids (Fig. 4a–d), whereas very few cells were attached to the endothelia of the vessels within the Glisson's sheath (i.e. hepatic arteries, and portal veins) (Fig. 4a). The cells attached to the central vein endothelia were positive for macrophage markers F4/80, CD11b, and LYVE-1 (Fig. 4e–g). However, by day 7, multiple granular staining cells translocated into the hematopoietic foci in the periportal regions (Fig. 4h–k). In contrast, very few cells were attached to the central vein on day 7 (Fig. 4h).

Recruitment of PKH26 labeled blood monocytes into the liver for hematopoiesis

In order to elucidate the source of recruited cells, PKH26 labeled peripheral blood monocytes from either PHZ-treated or non-treated mice were transfused in various combinations as shown in Figure 2. Four hours after transplantation, PKH26 labeled monocytes were observed in all samples regardless of the donor and recipient combination (Fig. 5a–c) though the degree of recruitment was significantly greater when the recipients had been treated with PHZ (S to P and P to P) (Fig. 6a). Three days after transplantation, only the PHZ treated recipients (S to P and P to P) showed hematopoiesis (Fig. 5d–f). No hematopoietic foci formation was noted in P to S or S to S samples. The hematopoietic foci in both samples contained PKH26 labeled cells. When double stained with CD45, a hematopoietic cell marker, PKH26 positive cells were in close contact with CD45 positive cells (Fig. 5g–i). The percentage of foci containing the labeled cells was significantly higher in P to P samples than in S to P samples (Fig. 6b).

Increased expression of chemokines and their receptors

Chemokines are known to be involved in monocyte recruitment. (Rollins, 1997). In order to determine whether the infiltration of blood monocytes into

Fig. 3. Induction of extra-medullary hematopoietic foci in the liver by PHZ. **a** and **b**: Hematoxylin-eosin staining of paraffin sections of the livers from PHZ-treated mice on day 4 (**a**) and day 7 (**b**). Arrows show hematopoietic foci in the periportal regions. **c**: An electron micrograph of a day 7 liver. Hematopoietic foci consist mostly of erythroid cells. Arrowheads show endothelial cells that have surrounded the hematopoietic foci. Bars: 30 μ m (**a**, **b**), 5 μ m (**c**)

hematopoietic foci was triggered by some chemokines, we analyzed the expression of chemokines by semiquantitative RT-PCR. Semi-quantitative RT-PCR of days 2, 4 and 7 livers revealed an increase in CCL-2 and CCL-5 along with their receptors CCR-1, CCR-2, CCR-3, CCR-5 (Fig.7). An increase in the expression of chemokine CX3CL-1 and its receptor CX3CR-1—which are known to be involved in the maintenance of tissue

resident macrophages (Geissmann *et al.*, 2003)—was also noted.

In situ hybridization of chemokine CCL-2

In situ hybridization of CCL-2 was performed to identify which cells produced these chemokines and receptors. In normal controls, no expression of CCL-2 was detected

Fig. 4. Intravital lectin labeling and immunostaining of hematopoietic livers with various macrophage markers. **a:** Numerous round cells which stained granular to FITC-RCA-I (arrows) adhering to the central veins (CV) and portal areas of the liver on day 4, whereas very few lectin staining cells can be identified in the vessels within the Glisson's sheath (GS). **b–d:** Larger magnification of FITC-RCA-I staining cells (arrows) attached to the central vein on day 4. Type IV collagen (**e**) was stained to delineate the vascular structure. Staining of F4/80 (**e**), CD11b (**f**) and LYVE-1 (**g**) for day 4 samples. **h:** Low magnification of RCA-I stained BM on day 7. Arrows show hematopoietic foci. Note that no lectin staining cells are bound to the CV endothelia. **i–k:** High magnification of RCA-I (**i**), type IV collagen (**j**), and merged (**k**) staining images on day 7. RCA-I staining cells (arrowheads) reside in the hematopoietic foci (dotted circle). Bar: 25 μ m (**a**, **h**), 50 μ m (**b–g**, **i–k**).

Fig. 5. Migration of PKH26 positive cells to the liver during extramedullary hematopoiesis. **a–c:** FITC-RCA-I (green) and PKH26 (red) positive cells (arrowheads) have infiltrated the liver 4 h after transplantation in a P to P combination. Bar: 75 μ m. **d–f:** Hematopoietic foci of P to P samples, 3 days after transplantation. PKH26 (red) positive cells (arrowheads) accumulate in type IV collagen (blue) lined hematopoietic foci. **g–i:** Staining of CD45 (arrowheads) and PKH26 (arrow) on P to P samples. Bars: 50 μ m (d–i)

Fig. 6. Quantification and distribution of PKH26 positive cells in the liver. **a:** The number of PKH26 cells per field that were recruited into the liver (cells per X200 magnified field). Bars indicate standard errors of the means. Asterisks: $p < 0.01$ compared with S to S. **b:** The percentage of foci that contained PKH26 positive cells in S to P and P to P samples. Bars indicate standard errors. Asterisk: $p < 0.01$

Fig. 7. Semi-quantitative PCR reaction of chemokines and chemokine receptors. PCR reaction of chemokine receptors CCR-1, CCR-2, CCR-3, and CCR-5 and their ligands. CCL-2 and CCL-5 have increased dramatically with hematopoiesis compared to controls. CX3CL-1 and its receptor CX3CR-1 also showed an increase in expression.

Fig. 8. *In situ* hybridization of chemokines CCL-2 and CCL-5. **a:** *In situ* hybridization of day 4 liver for CCL-2. **b:** ISH of day 7 livers for CCL-2. **c:** A serial adjacent section of **b** immuno-stained for F4/80, Brown stainings by DAB indicate the positivity for the chemokine mRNA on *in situ* hybridization sections (arrows). Brown stainings for DAB indicate positivity for F4/80 by immunohistochemistry (arrowheads). Counterstaining with hematoxylin. Note that the CCL-2 positive cells on **b** are positive for F4/80 on **c**. Bar: 10 μ m

(data not shown). In the day 4 liver, CCL-2 (Fig. 8a) were positive in the central vein endothelia. On day 7, CCL-2 production was noted on cells comprising the hematopoietic foci and endothelial cells (Fig 8b). Adjacent immunostained paraffin sections showed that F4/80 positive macrophages also produced CCL-2 on day 7 (Fig. 8 b, c). CCL-2 expressions were not noted on the sinusoidal endothelial cells surrounding the hematopoietic foci.

Discussion

This study aimed to identify morphological and functional changes of the adult liver during extramedullary hematopoiesis. Using PHZ-treated splenectomized mice, we elucidated the role of recruited monocytes at the onset of and during extramedullary hematopoiesis in the liver. Our results identify and reveal characteristics of the extramedullary hematopoietic milieu of the liver which are comparable to that of the adult bone marrow yet are distinctive on their own.

We showed that monocytes of extra-hepatic origin are recruited into the liver to function as central macrophages. Prior to hematopoietic foci formation, numerous monocytes attach to the central vein endothelia and translocate to the proximity of the portal veins in the liver. Hematopoiesis induced by PHZ has been known to result in foci formation in the periportal regions (Vidal-Vanaclocha *et al.*, 1990), with which our results concur.

A thin layer of endothelial cells surrounded most of the hematopoietic foci, which suggested that hematopoietic foci formed primarily intra-luminally within the expanded sinusoids. Also, *in situ* hybridization analyses confirmed that the central vein endothelia produced the monocyte attracting chemokines CCL-2 and CCL-5 on day 4 prior to hematopoietic foci formation. These monocytes were thought to migrate in a retrograde direction from the central veins towards the portal areas for hematopoiesis. Transplanted PKH26-labeled peripheral blood monocytes were incorporated in the hematopoietic foci and were in close contact with hematopoietic cells, seemingly nursing these cells until maturation. Some reports have identified lymphoid cells as migrating into the liver during extramedullary hematopoiesis (Mori *et al.*, 1987); however, reports like ours that specify the direct migration and involvement of extra-hepatic monocytes are without precedent. The exact origin of these peripheral blood monocytes and their precise mechanism of activation to induce and support hematopoiesis remain unclear and should be further investigated.

Not only the recruited monocytes but also the resident cellular components were necessary for extramedullary hematopoiesis. The PKH26 transplantation showed that the PHZ-treated liver increased its potential to recruit peripheral monocytes. Still, not all hematopoietic foci contained recruited PKH26 positive cells. Moreover, no foci formed in P to S or S to S transplants, showing that recruited monocytes alone cannot initiate hematopoiesis. Semi-quantitative PCR showed an increase in CCL-2 and

CCL-5, which mainly involve the recruitment of activated monocytes during various processes such as inflammation (Mantovani *et al.*, 2004). On the other hand, we also noted an increase in CX3CL-1 and its receptor that homeostatically recruit resident macrophages (Geissmann *et al.*, 2003). This suggested that both recruited monocytes and resident macrophages participated in liver hematopoiesis.

Our data also indicated that chemokines may be involved in later stages of extramedullary hematopoiesis. Even after initial monocyte recruitment, PCR data showed a persistent chemokine production. Day 7 *in situ* hybridization confirmed that F4/80 positive macrophages in the hematopoietic foci produced CCL-2 which accentuated the relevance of these chemokines during hematopoiesis. However, the degree and the precise mechanisms through which these chemokines act need further investigation. Chemokine CCL-3 (MIP-1 α) and its receptors act as regulators of bone marrow hematopoiesis (Broxmeyer and Kim, 1999). The intense expression of CCL-2 and CCL-5 within the hematopoietic foci suggests that these chemokines and their receptors may play a role in the regulation of extramedullary hematopoiesis.

In conclusion, the recruitment of extra-hepatic monocytes is a vital process during extramedullary hematopoiesis of the liver. The local and extra-hepatic monocytes compromise a milieu for extramedullary hematopoiesis in the liver. Ontogenetically, macrophages carry primitive characteristics and are the first cells that respond to pathological conditions such as inflammation (Anastassova-Kristeva, 2003). Indeed, macrophages play a pivotal role in recruiting immature cells during angiogenesis and tumor metastasis (Lewis and Pollard, 2006). They have thus been recently recognized as an important participant of the pre-metastatic niche for tumor progression and invasion (Mantovani *et al.*, 2006). Assuming that monocyte activation preceded hematopoiesis, our observations provide additional insight into characterizing these cells as relevant supporting cells in extramedullary hematopoiesis.

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