Aminopeptidase N/CD13 Regulates the Fetal Liver Microenvironment of Hematopoiesis

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Fetal liver (FL) hematopoiesis is thought to be important for expanding the cell number during ontogeny. In order to investigate the cellular interaction molecules among FL stromal and hematopoietic cells, we established a monoclonal antibody, Ndk-10, that reacts with FL stromal cells but not with dish non-adherent cells. When Ndk-10 was added to an FL stromal and hematopoietic cell-coculture, it inhibited the survival of c-kit+ cells. The inhibitory activity of Ndk-10 was also observed in the fetal liver organ culture. The Ndk-10 recognized a 150 kD molecule in the adherent cells of FL and kidney, and the N-terminal amino acid sequence was identical to that of mouse aminopeptidase N/CD13. The peptidase activity of CD13 was inhibited by Ndk-10, and addition of its specific inhibitor resulted in the same inhibitory activity as Ndk-10. We propose that aminopeptidase N/CD13 is a critical molecule that regulates the survival of c-kit+ cells in the FL microenvironment.

key words  fetal liver; microenvironment; CD13; aminopeptidase; hematopoiesis; stromal cell

Hematopoietic stem cells (HSCs) are defined by their ability to give rise to both new stem cells and all blood cells. During mouse ontogenesis, definitive hematopoiesis initially occurs in the aorta-gonad-mesonephros as early as gestational day 10 (E10).1) HSCs migrate to the fetal liver (FL) around E12, and exist there during the late gestational period.2,3) After birth, HSCs migrate to the bone marrow (BM) and are present there for life. It is speculated that the late gestational period is important for expanding the number of hematopoietic cells. It has been reported that HSCs derived from FL have superior repopulation and developmental capacities than other hematopoietic cells such as bone marrow cells,4,5) indicating that the FL microenvironment induces active hematopoiesis.

The microenvironment is essential for the generation of hematopoietic cells and consists of stromal cells. Studies of the hematopoietic microenvironment using BM stromal cells have been reported by several investigators. Dexter et al. established long term BM cultures as a model system.6) Using the system, several molecules that regulate hematopoietic cell differentiation were identified.7,8) For instance, Miyake et al. reported that cell–cell interactions through VLA-4 and VCAM-1 are important for lymphohematopoiesis in BM.9)

The role of FL stromal cells in the fetal hematopoiesis have also been investigated.10—12) Kinoshita et al. reported that the FL-derived stromal layer comprises mostly fetal hepatic cells, and these cells support the expansion of hematopoietic cells. Hepatic differentiation and fetal hematopoiesis are inversely correlated during ontogenesis.13,14) Although many studies have examined the hematopoietic microenvironment, the molecular mechanisms of the FL microenvironment remain unclear.

In the present paper, we describe the establishment of a novel monoclonal antibody (mAb), Ndk-10, that detects a surface molecule on FL stromal cells. Ndk-10 inhibits the survival of c-kit+ hematopoietic cells on FL stromal cells. The Ndk-10 reactive molecule is identified as mouse aminopeptidase N/CD13 (APN/CD13). These results suggest that the APN/CD13 is an important element of the hematopoietic microenvironment in FL.

MATERIALS AND METHODS

Mice  All procedures used on experimental animals were approved by the experimental Animal Care and Use Committee at Graduate School of Pharmaceutical Sciences, Osaka University. Specific pathogen-free C57BL/6 mice aged 6 to 8 weeks were purchased from Charles River Japan (Yokohama, Japan). The mice were mated at night, and females were examined the next morning. The day on which a vaginal plug was found was considered day 0 of embryonic development. Sprague-Dawley rats were purchased from CLEA Japan (Tokyo, Japan).

Antibodies and Reagents  The rat mAb to mouse c-kit (ACK2) was a gift from Dr. S.-I. Nishikawa (Kyoto University, Kyoto, Japan),13) and was labeled with FITC in our laboratory. FITC-conjugated goat anti-rat IgG, biotin-conjugated goat anti-rat IgG (H+L), and the rat mAb to mouse CD13 (R3-242) were purchased from ICN pharmaceuticals (Aurora, OH), Cedarlane (Ontario, Canada), and BD Pharmingen (Franklin Lakes, NJ, U.S.A.), respectively. Ubenimex (Bestatin, MW: 308.4, a substance produced by Streptomyces olivoreticuli), an inhibitor of APN/CD13 and aminopeptidase B, was purchased from Wako Pure Chemicals (Osaka, Japan).

Preparation of Fetal Liver and Hematopoietic Cell Fractions  FL stromal cells were prepared from the liver of gestational day 14 (E14) embryos as follows. FL lobes were gently washed in phosphate-buffered saline (PBS) for 15 min, and treated with 0.05% trypsin and 0.02% EDTA in PBS for 30 min at 37 °C. The lobes were gently pipetted, and filtered through a nylon mesh to remove undigested fragments, and then cultured in Dulbecco’s modified Eagle’s medium (Sigma Chemical, St. Louis, MO, U.S.A.) containing 10% horse serum (Invitrogen, Carlsbad, CA, U.S.A.) and kanamycin (100 mg/l, Meiji Seika, Tokyo, Japan). Twelve hours later, non-adherent cells were collected by gentle swirling of the culture dishes with a sufficient volume of cul-
ulture medium. The non-adherent fraction of FL cells included 20—30% c-kit+ cells and they died within 24 h of culture without any additional cells or growth factors. The adherent and non-adherent fractions of FL cells are called FL stromal cells and hematopoietic cells, respectively, hereafter.

**Flow Cytometry** About 5×10^5 cells were incubated with hybridoma culture supernatants or about 1 μg/ml purified mAbs in PBS containing 1% fetal calf serum (FCS, Boehringer Mannheim, Acton, MA, U.S.A.) and 0.1% sodium azide for 30 min on ice. The cells were washed once with the buffer and incubated with second antibodies for 30 min on ice, and rendered to flow cytometry. Flow cytometric profiles were analyzed with a FACSCalibur analyzer and CELLQuest software (Becton Dickinson Immunocytometry Systems, Mountain View, CA, U.S.A.).

**Cell Coculture System** 5×10^5 FL stromal cells were preincubated to adhere on a 24-well plate (Nunc, Roskilde, Denmark) for 48 h. Then 1×10^5 FL hematopoietic cells were added to each well and cultured in RPMI 1640 medium (Sigma Chemical) containing 10% FCS and kanamycin (100 μg/ml). Four days later, non-adherent cells were recovered, and the number of c-kit+ cells was determined. During this coculture period, various mAbs (10 μg/ml) or Ubenimex (3.2—320 μM) were added. In some experiments, frequencies of apoptotic cell death were determined by staining the cells with annexin V and propidium iodide (PI) (using apoptosis detection kit, product of BioVision, Mountain View, CA, U.S.A.) according to the methods of Vermes et al. [16].

**Fetal Liver Organ Culture System** Fetal liver organ culture (FLOC) was performed according to the methods of Owen et al. [17] and Ceredig et al. [18]. Gestational day 12 (E12) fetal livers were taken and sliced into 4 pieces with a razor blade. The pieces were then transferred onto Nucleopore membrane filters (Nuclepore-Corning, Pleasanton, CA, U.S.A.), and FLOC was performed with or without mAb (10 μg/ml) or Ubenimex (320 μM). Culture medium is the same as for cell co-culture system. Six days later, cultured fetal livers were recovered, gently homogenized, filtered through a nylon mesh, and dish-non-adherent single cell suspensions were prepared. Frequencies of c-kit+ cells were determined by FACS.

**Generation of Monoclonal Antibodies** mAbs were established according to the usual method. Briefly, intact FL stromal cells were repeatedly injected intraperitoneally into rats. Three days after the last injection, the rats were sacrificed under anesthesia, and splenocytes were fused with myeloma cells, P3X63Ag8U.1 (P3U1). Strategies for mAb selection are described in the results section.

**Immunohistochemical Staining** Cryostat sections of various tissues were fixed in acetone for 10 min and then incubated with 5% bovine serum albumin (Sigma Chemical) in PBS. The sections were incubated for 1 h at 37 °C with monoclonal antibodies (30 μg/ml), followed by further incubation for 30 min with biotinylated goat anti-rat IgG (H+L), and then with 0.3% H2O2 (with 0.1% NaIO3) in PBS, to remove endogenous peroxidase activity. The avidin–biotin reaction was achieved by using Vectastain ABC elite kit (Vector Laboratories, Burlingame, CA, U.S.A.). Visualization of peroxidase activity was performed by incubating the sections with an equivalent mixture of 1 mg/ml DAB (3,3'-diaminobenzidine, Dojindo, Kumamoto, Japan) in 0.1 M Tris–HCl and 0.02% H2O2. The sections were counterstained with hematoxyline.

**Immunoprecipitation and Immunoblotting** FL stromal cells and various tissues were homogenized in ice-cold lysis buffer (1% NP40, 150 mM NaCl, 50 mM Tris (pH 7.4), 5 mM EDTA, and 1 mM benzamide), and the supernatants were collected after centrifugation. The cell lysates were precleared with protein G-Sepharose (Amersham Pharmacia Biotech, Arlington Heights, IL, U.S.A.) for over 1 h at 4 °C. For immunoprecipitation, the lysates were incubated with 5 μg mAb (Ndk-10 or R3-242) for over 2 h at 4 °C. After washing twice with 1 ml lysis buffer, immunoprecipitates were resolved by 7.5% SDS-PAGE. Proteins in the immunoprecipitats were transferred from the SDS-polyacrylamide gel to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, U.S.A.), blocked with 5% skim milk in TBS-T (20 mM Tris–HCl (pH 8.0), 137 mM NaCl, and 0.1% Tween 20) and incubated with primary antibody (Ndk-10) at room temperature for 1 h, and then washed three times with TBS-T. To detect antibody binding, horseradish peroxidase (HRP)-conjugated anti-rat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in TBS-T was incubated at room temperature for 1 h. After washing three times in TBS-T, the bound HRP conjugates were visualized with enhanced chemiluminescent reagent (Wako Pure Chemicals).

**Amino Acid Sequencing** A kidney lysate was immunoprecipitated with Ndk-10, and resolved by SDS-PAGE as described above. Proteins in the immunoprecipitate were transferred from the SDS-polyacrylamide gel to a PVDF membrane (BIO-RAD, Hercules, CA, U.S.A.). The membrane was stained with Coomassie R-250, the 150 kD band was excised, and the N-terminal sequence was determined by Nippi Co. Ltd. (Tokyo, Japan).

**Peptidase Activity** The peptidase activity of cell surface APN/CD13 was measured according to the method of Ashmun et al. [19]. Intact FL stromal cells were incubated at 37 °C for 1 h in PBS containing 6 mM alanine-p-nitroanilide (Sigma Chemicals), a substrate for APN/CD13. After centrifugation at 4 °C, the absorbance at 405 nm of the cell free supernatants was measured to detect free p-nitroaniline liberated by cleavage of the substrate by APN/CD13. All measurements were made in triplicate. FL stromal cells were preincubated with Ndk-10 or control IgG (20 μg/ml) for 12 h.

**RESULTS**

**Production of mAb** mAbs were screened by the following two-step selection. First, mAbs that stained FL stromal cells were selected. Among them, mAbs that stained FL hematopoietic cells were discarded. Because we intended to examine molecules participating in the FL microenvironment, we chose mAbs that reacted only with the stromal cell fraction of FL. Figure 1 shows the flow cytometric patterns of 3 typical clones among the 14 selected clones. These clones reacted only with the adherent FL stromal cell fraction, but not with FL hematopoietic cells. The isotype of Ndk-10 was IgG2b.

**Inhibition of c-kit+ Cell Survival by mAb** To study the role of the antigen recognized by these mAbs in the FL microenvironment, we established an in vitro culture method to detect the interaction between FL stromal cells and
hematopoietic cells. Hematopoietic cells were cultured on
0—5\times10^4 FL stromal cells. Four days later, the number of c-
kit^+ cells was determined. c-kit is widely known as a marker
molecule of hematopoietic progenitor cells.\(^{20}\) As shown in
Fig. 2, c-kit^+ hematopoietic cells survived on FL stromal
cells in proportion to the number of FL stromal cells. These
results suggest that FL stromal cells can support hematopoi-
etic cell survival.

To examine the inhibitory activity of the mAbs on
hematopoietic cell survival, 14 mAbs were added to the co-
culture. As shown in Fig. 3A, several mAbs inhibited the sur-
vival of c-kit^+ cells. Among them, Ndk-10 showed the
strongest inhibition of c-kit^+ cell survival on FL stromal
cells. The inhibitory activity of Ndk-10 was also observed in
the FLOC system (Fig. 3B).

**Immunohistochemical Analysis of Ndk-10 Antigen** To
examine the expression pattern of the Ndk-10 antigen, we
performed an immunohistochemical analysis of various fetal
and adult tissues. Cryostat sections of E14 embryonic organs
were stained with Ndk-10 (Figs. 4A—E). In fetal tissues,
Ndk-10 antigen was expressed strongly in the liver and
slightly in the thymus. The expression of the Ndk-10 antigen
in various tissues from 6-week-old C57BL/6 mice is shown
in Figs. 4F—K. The Ndk-10 antigen was detected in lym-
phoid tissues such as the spleen (Fig. 4J) and thymus (Fig.
4K). Because we checked that lymphocytes did not express
the Ndk-10 antigen by flow cytometry (data not shown), the
distribution was thought to be due to expression on stromal
cells. On the other hand, in non-hematopoietic tissues, strong
expression was observed in the kidney, especially in renal
tubular epithelial cells. Other tissues displayed weak expres-
sion. Because the Ndk-10 antigen was detected in various

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**Fig. 1. Establishment of Monoclonal Antibodies Recognizing FL Adherent Cells**

FL-derived adherent (stromal) and non-adherent (hematopoietic) cells were stained
with mAbs together with FITC-conjugated goat anti-rat IgG. The viable cell fraction
was gated and single-color flow cytometric patterns are shown. Thin lines indicate
background staining (second Ab alone) (X-axis: log fluorescence intensity, Y-axis: cell
counts/10^4 cells).

**Fig. 2. Survival of Hematopoietic Cells Is Dependent on Fetal Liver Stromal Cells**

FL-derived stromal cells (0—5\times10^4 cells/well) and FL-derived non-adher-
ent (hematopoietic) cells (\(1\times10^5\) cells/well, \(2\times10^5\) cells/well, \(1\times10^6\) cells/well)
were cocultured. c-kit^+ cells in the FL-derived non-adherent fraction were 18% in this
experiment. Four days later, non-adherent cells were collected and counted. Recovered
cells were stained with FITC-conjugated anti-c-kit mAb (ACK2) and the percentage of
c-kit^+ cells was measured by flow cytometry. The results indicate the number of c-kit^+
cells (\times10^4 cells) (Y-axis).

**Fig. 3. Survival of c-kit^+ Cells Is Inhibited by mAb**

(A) Hematopoietic cells derived from FL were cultured on FL stromal cells in the
presence of mAbs (10 \(\mu\)g/ml). Four days later, floating cells were collected and
counted. The cells were stained with FITC-conjugated anti-c-kit mAb (ACK2) and the
percentage of c-kit^+ cells was measured by flow cytometry. The results are given as
percent recovery compared with medium only. The number of c-kit^+ cells when cul-
tured in medium only was 5.4—19.6\times10^4 in each experiment. (B) Effect of Ndk-10
was examined by FLOC system. E12 FLs were cultured for 6 d on Nuclepore mem-
brane with or without Ndk-10 (10 \(\mu\)g/ml) or Ubenimex (320 \(\mu\)M). Number of c-kit^+ cells
were determined by FACS. Statistical analysis was performed by Student’s t-test
(\(*1: p>0.002, *2: p<0.001\) compared with control IgG).
hematopoietic tissues, such as FL, spleen and thymus, the antigen is thought to be an important molecule for maintaining the hematopoietic microenvironment. Since it is also detected in the kidney, the Ndk-10 antigen may have function(s) other than the maintenance of the hematopoietic microenvironment.

In liver, a supportive function of hematopoietic cells exists only during the fetal period. We then examined the expression patterns during liver ontogenesis. The Ndk-10 antigen was observed in the liver from E12 to adult (Figs. 4L—Q). The Ndk-10 antigen was strongly expressed in the liver in the fetal period, and the expression was lower in neonatal and adult livers. It is interesting to note that the intensity of Ndk-10 antigen expression correlates well with the supportive function of hematopoietic cells in liver.

Identification of Ndk-10 Antigen

In order to characterize Ndk-10 antigen, cell lysates from FL stromal cells were immunoprecipitated and immunoblotted with Ndk-10. As shown in Fig. 5A, Ndk-10 precipitated an approximately 150 kD protein from FL stromal cells. Next, we examined the tissue distribution of the Ndk-10 antigen by immunoblot analysis. Fig. 5B indicates the expression of Ndk-10 antigen in various tissues. Except for FL stromal cells (positive control), the 150 kD protein was detected only in FL and kidney. Especially, the kidney showed the strongest expression among the tissues examined. Therefore, we performed amino acid sequence analysis of the Ndk-10 antigen immunoprecipitated from kidney. It was found that a 14-amino acid sequence at the N-terminal of the Ndk-10 antigen was identical to that of mouse APN/CD13. Then, we immunoprecipitated the 150 kD protein from FL adherent cell lysates with a commercially available anti-mouse CD13 mAb (R3-242), and immunoblotted with Ndk-10. As shown in Fig. 5C, Ndk-10 as well as the anti-CD13 mAb immunoprecipitated the 150 kD protein, indicating that the Ndk-10 antigen is mouse APN/CD13.

Analysis of CD13 Peptidase Activity on FL Stromal Cells

The peptidase activity of cell surface CD13 on FL stromal cells was measured by a spectrophotometric assay. When alanine-$p$-nitroanilide was incubated with FL stromal cells, $p$-nitroaniline liberated by cleavage of the substrate was detected (O.D. 405 nm $= 1.03 \pm 0.22$ was obtained as com-
pared with the cell-free negative control). Because this assay detects the CD13 peptidase activity specifically, the result suggests that CD13 on FL stromal cells has peptidase activity. As shown in Fig. 6, when Ndk-10 was added to FL stromal cells, the peptidase activity was significantly inhibited, indicating that Ndk-10 downregulates the peptidase activity of CD13 on FL stromal cells.

**Effect of Ubenimex on the Survival of Fetal Liver Hematopoietic Cell Fraction**  The effect of Ubenimex, an inhibitor of APN/CD13, 21) on hematopoietic cell survival was examined. As shown in Fig. 7A, the recovery of c-kit+ cells decreased when Ubenimex was added to the culture. Similar to the experiment with Ndk-10, the percentage of c-kit+ cells also decreased (data not shown). The inhibitory effect of Ubenimex was also observed in the FLOC system (Fig. 3B). As shown in Fig. 7B, the addition of Ubenimex increased the cell death. Apoptotic cell death was higher in the group of Ubenimex (41—43%) than control group (21—27%). Since the APN/CD13 is not expressed on the surface of non-adherent cell fraction of FL cells, some unknown mechanisms are operating in the induction of cell death by anti-APN/CD13 or Ubenimex.

Taken together, the results suggest that the peptidase activity of cell surface APN/CD13 is important for the maintenance of c-kit+ hematopoietic cells.

**DISCUSSION**

Addition of a newly established mAb directed to FL stromal cells, Ndk-10, to the coculture of FL adherent (stromal) cells and non-adherent (hematopoietic) cells decreased the recovery of c-kit+ cells (Fig. 3A). In the coculture system, the FL-derived, non-adherent cell fraction was used as a hematopoietic cell source. Although the fraction contains cell types other than hematopoietic cells, the cells in the fraction could not survive alone and did not express the Ndk-10 antigen. Thus, it is clear that FL stromal cells and the cell surface Ndk-10 antigen are important for the maintenance of c-kit+ cells. The effect of Ndk-10 was confirmed by the FLOC system (Fig. 3B). We then examined the expression of the Ndk-10 antigen on various tissues by immunohistochemical (Fig. 4) and immunoblotting (Fig. 5) analyses, and finally
the amino acid sequence data definitively revealed that the Ndk-10 detects CD13 molecule. Because E14 FL adherent cells expressed albumin when they cultured (4 d, data not shown), CD13 expressing FL adherent cells are thought to be fetal hepatocytes or its precursors.

APN/CD13 is one of the membrane-bound metalloproteinases that preferentially degrade proteins with an N-terminal neutral amino acid. In humans, CD13 is expressed in various myeloid lineage cells, including monocytes, granulocytes, and immature myeloid cells, and is used as a myeloid lineage marker.2) But in mouse hematopoietic cells, CD13 is expressed only in mature macrophages and dendritic cells.23,24) This fact enabled us to investigate the role of CD13 on stromal cells. In non-hematopoietic cells, CD13 is expressed on epithelial cells of the intestine and kidney, synapic membranes in the central nervous system, fibroblasts, endothelial cells, and some human tumor cells.2) It has also been reported that CD13 is expressed in human BM stromal cells.2) However, little is known about the role of CD13 molecule on stromal cells.

In the hematopoietic microenvironment, it is known that stromal cells are an important element in the maintenance and differentiation of hematopoietic cells.2—13) In recent years, several reports have indicated that some proteases perform essential roles in hematopoesis. For instance, Heissig et al. reported that MMP-9 induced in BM cells releases a soluble kit-ligand, permitting the transfer of endothelial cells and differentiation of hematopoietic cells.7—11) In recent years, several reports have indicated that some proteases perform essential roles in hematopoesis. For instance, Heissig et al. reported that MMP-9 induced in BM cells releases a soluble kit-ligand, permitting the transfer of endothelial cells and hematopoietic stem cells from the quiescent proliferative niche.21) Levesque et al. reported that VCAM-1 expressed on BM stromal cells is cleaved by neutrophil proteases.22) These reports indicate that the enzymatic digestion of membrane bound molecules might be a key step in hematopoesis on stromal cells. In the present study, we found that APN/CD13 regulates the maintenance of hematopoietic cells on FL stromal cells. Similar to the reported proteases, CD13 may function as an essential protease for regulating the hematopoietic microenvironment.

Known substrates of CD13 are enkephalines, opioid peptides,23) interleukin-1β, and extracellular matrix proteins such as collagen type-IV.22) Mishima et al. reported that the cell surface CD13 appear to allow prevents human leukemic cells to resist endothelial IL-8 induced cell death.28) Anti-APN/CD13 induced cell death (Fig. 7B) may be a result of inhibition of APN/CD13-induced degradation of some apoptosis-blocking molecules, such as IL-8 in the case of human leukemias;23) on the surface of FL stromal cells. Because the Ndk-10 was less effective at the dose tested than Ubenimex (see Fig. 3B), apoptotic cell death of the Ndk-10 was less effective at the dose tested than Ubenimex.2) However, the molecular mechanisms of anti-APN/CD13 induced cell death are not yet known and this should be clarified in the future experiments.

We report here that the Ndk-10 blocks the peptidase activity of CD13, and also an APN/CD13-specific inhibitor, Ubenimex, decreases cell recovery. Judging from these results, it is suggested that the enzymatic activity of APN/CD13 is necessary for the maintenance of hematopoietic cells in the FL microenvironment (Figs. 6, 7). However, the natural substrate of CD13 in hematopoietic cell survival remains unknown. Tani et al. reported that CD13 has a chemotactic activity for T lymphocytes, and the activity is dependent on its peptidase activity.29) The report proposed the possibility that CD13 has function(s) other than cleaving some substrates.

CD13 is also expressed in other hematopoietic tissues, including spleen, thymus (Fig. 4), and BM stromal cells.30) We confirmed that CD13 is also important for BM and thymus stromal cell functions, using a coculture method similar to FL coculture or fetal thymus organ culture methods (unpublished data). These results suggest that CD13-mediated cellular interactions are common events during various developmental stages of hematopoietic cells. It is interesting to note that the distribution of CD13 was altered during ontogenesis (Fig. 4). Therefore, there is a possibility that CD13 or its substrate may play different roles during development than in adult.

Using the newly established Ndk-10, we found that cell surface APN/CD13 plays an important role in FL hematopoesis that is dependent on its peptidase activity. Our findings provide a novel model that may explain the molecular mechanisms of cell survival and development of hematopoietic cells in the FL microenvironment.

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REFERENCES