CD45 tyrosine phosphatase inhibits erythroid differentiation of umbilical cord blood CD34+ cells associated with selective inactivation of Lyn

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CD45 is a membrane-associated tyrosine phosphatase that dephosphorylates Src family kinases and Janus kinases (JAKs). To clarify the role of CD45 in hematopoietic differentiation, we examined the effects of anti-CD45 monoclonal antibody NU-LPAN on the proliferation and differentiation of umbilical cord blood CD34+ cells. NU-LPAN showed a prominent inhibition of the proliferation of CD34+ cells induced by the mouse bone marrow stromal cell line MS-5 or erythropoietin (EPO). However, NU-LPAN did not affect the proliferation induced by interleukin 3. NU-LPAN also inhibited MS-5–induced or EPO-induced erythroid differentiation of CD34+ cells. The cells stimulated with EPO in the presence of NU-LPAN morphologically showed differentiation arrest at the stage of basophilic erythroblasts after 11 days of culture, whereas the cells treated with EPO without NU-LPAN differentiated into mature red blood cells. The Src family kinase Lyn and JAK2 were phosphorylated when erythroblasts obtained after 4 days of culture of CD34+ cells in the presence of EPO were restimulated with EPO. Overnight NU-LPAN treatment before addition of EPO reduced the phosphorylation of Lyn but not that of JAK2. Simultaneously, the enhancement of Lyn kinase activity after restimulation with EPO was reduced by NU-LPAN treatment. These results indicate selective inactivation of Lyn by CD45 activated with NU-LPAN and could partly explain the inhibitory mechanism on erythropoiesis exhibited by EPO. These findings suggest that CD45 may play a pivotal role in erythropoiesis.

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Materials and methods

Cells

The UCB samples were obtained from clamped umbilical cords at full-term normal pregnancies. Approval was obtained from the institutional review board at the Kurashiki Medical Center for these studies. Informed consent was provided according to the Declaration of Helsinki. Mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque (Amerham Pharmacia Biotech, Buckinghamshire, United Kingdom). Selection of CD34+ cells was achieved with a magnetic activated cell sorting (MACS) CD34+ isolation kit and a mini-MACS column (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Briefly, Ficoll-Paque–separated cells were first labeled with hapten-conjugated anti-CD34 primary antibody (QBEND/10) and then mixed with magnetic beads conjugated to anti-hapten secondary antibody. These magnetically labeled cells were separated into CD34+ and CD34− populations on a mini-MACS column. The column separation steps were repeated.

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twice to obtain a highly enriched CD34+ population. Purity of the CD34+ cells was confirmed by flow cytometry using fluorescein isothiocyanate (FITC)-conjugated anti-CD34 antibody (8G12; BD Biosciences, San Jose, CA). Populations with higher than 90% purity of CD34+ cells were used for our experiments. Mouse bone marrow stromal cell line MS-52 was a generous gift from Dr K. J. Mori (Niigata University, Niigata, Japan) and was maintained in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Tokyo, Japan), 100 U/mL penicillin, and 50 μg/mL streptomycin (Sigma, St Louis, MO).

Coculture of CD34+ cells with MS-5 cells

MS-5 cells were plated at 1 × 104 cells/mL in 24-well tissue culture plates (Corning, Corning, NY) 1 week before coculture was started. CD34+ cells were resuspended into RPMI 1640 medium supplemented with 20% BIT9500 serum substitute (Stem Cell Technologies, Vancouver, BC, Canada) and antibodies (100 U/mL penicillin and 50 μg/mL streptomycin) at a density of 1 × 106 cells/mL with or without the addition of 20 μg/mL anti-CD45 monoclonal antibody NU-L_PAN (Nichirei, Tokyo, Japan). The hybridoma culture supernatant was used as antibody source. The culture supernatant of MS-5 cells was replaced with CD34+ cell suspensions, and then the cells were cultured at 37°C in a humidified 5% CO2 atmosphere for the subsequent 2 weeks. Half of the medium was replaced with fresh medium on day 7. After 2 weeks, nonadherent cells were counted and harvested. Adherent cells were also harvested at the same time by detachment with trypsin-EDTA (ethylenediaminetetraacetic acid; Invitrogen) and mixed with nonadherent cells. Wright staining was performed on cytosin smears for morphologic examination and the remaining cells were tested for colony formation. To confirm the specificity of the NU-L_PAN culture supernatant, protein G-Sepharose (Amersham Pharmacia Biotech)–purified NU-L_PAN and MOPC-21 immunoglobulin class–matched antibody (ICN Pharmaceuticals, Costa Mesa, CA) were used. Purified NU-L_PAN was capable of reproducing the effect of the NU-L_PAN culture supernatant, and MOPC-21 showed no effect on the proliferation and differentiation of CD34+ cells (data not shown).

Cytokine stimulation

CD34+ cells were resuspended into RPMI 1640 medium supplemented with 20% BIT9500 and antibiotics. The cells were plated at a density of 1 × 104 cells/mL for proliferation and colony formation assays, and at 1 × 105 cells/mL for flow cytometric analysis and determination of hemoglobin concentration with or without the addition of NU-L_PAN. Stem cell factor (SCF), IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF; R & D Systems, Minneapolis, MN) were added to the cultures at a concentration of 100 ng/mL. EPO (Hayashibara Biochem Labs, Okayama, Japan) or thrombopoietin (TPO; R & D Systems) was added at concentrations of 5 U/mL and 50 ng/mL, respectively. After 5 to 11 days of culture, the number of cells was counted and cytosin smears were made for Wright staining.

Colony formation assay

Untreated CD34+ cells, cells after coculture with MS-5 or cells exposed to EPO were resuspended in Iscove modified Dulbecco medium (Invitrogen). Cell suspensions containing 500 to 2.5 × 106 cells in 100 μL volumes of medium were mixed with 1 mL cytokine-containing methylcellulose medium (MethoCult GF® H4435; Stem Cell Technologies). After 2 weeks of culture at 37°C in a humidified 5% CO2 atmosphere, colonies were counted and classified into granulocyte-erythrocyte-macrophage-megakaryocyte colony-forming units (CFU-GEMMs), granulocyte-macrophage colony-forming units (CFU-GMs), or erythrocyte burst-forming units (BFU-Es) under the inverted microscope.

Flow cytometry

Expression of CD34 and glycophorin A on EPO-stimulated cells was determined by flow cytometry on an EPICS XL (Beckman Coulter, Fullerton, CA) using FITC-conjugated anti-CD34 antibody 8G12 and phycoerythrin-conjugated anti–glycophorin A antibody (GA-R2, BD Pharmingen, San Diego, CA), respectively.

Determination of hemoglobin concentration

Hemoglobin concentrations of EPO-stimulated cells were determined by colorimetric assay with diaminofluorescein as described elsewhere.

Preparation of cells for Western blotting, immunoprecipitation, and in vitro kinase assay

CD34+ cells stimulated with 5 U/mL EPO for 4 days were cultured in RPMI 1640 medium supplemented with 20% BIT9500 for 18 hours with or without the addition of 20 μg/mL NU-L_PAN. The number of cells was adjusted to 0.8 to 2.5 × 106 cells/mL and 500 to 1000 μL aliquots of the cell suspensions were seeded into reaction tubes in triplicate. After 15 minutes of incubation at 37°C, cells were stimulated with EPO (final concentration of 5 U/mL) for 0, 5, or 15 minutes.

Western blotting

The EPO-stimulated cells were harvested and washed once with ice-cold washing buffer solution containing 50 mM Tris (tris(hydroxymethyl)amino-methane)–HCl (pH 7.4), 2 mM sodium orthovanadate, 100 mM sodium fluoride, and 1 mM (P-aminophenyl) methanesulfonyl fluoride hydrochloride. Cells were resuspended in 10 mL washing buffer solution and mixed with 10 μL sample buffer solution (4% sodium dodecyl sulfate [SDS], 20% glycerol, and 2 mM β-mercaptoethanol), and then heated at 100°C for 5 minutes for denaturation. Denatured samples were loaded onto 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Nitropure; Osmonics, Westborough, MA). The polyclonal antibodies rabbit anti-JAK2 (Upstate Biotechnology, Lake Placid, NY), rabbit antiphosphorylated JAK2 (Biosource International, Camarillo, CA), and rabbit anti-Lyn (Santa Cruz Biotechnology, Santa Cruz, CA) were used for the detection of JAK2, phosphorylated JAK2, and Lyn, respectively. A monoclonal mouse antiphosphorylated tyrosine antibody (4G10; Upstate Biotechnology) was used for the detection of phosphorylated Lyn. Polyclonal antibodies reactive against the respective primary antibodies conjugated to horseradish peroxidase (Dako, Glostrup, Denmark) were used as secondary reagents.

Immunoprecipitation and in vitro kinase assay

EPO-stimulated cells were harvested and washed once with ice-cold washing buffer solution. Pellets were lysed with 300 μL of a lysis solution containing 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, and 1 mM (P-aminophenyl) methanesulfonyl fluoride hydrochloride for 1 hour on ice. Lysates were centrifuged at 15 000g for 5 minutes at 4°C to remove nuclei and cell debris. Supernatants were incubated with a polyclonal rabbit anti-Lyn antibody for 3 hours at 4°C, and conjugates of antibody and Lyn proteins were coupled to protein G-Sepharose beads (Amersham Pharmacia Biotech) by incubation for 16 hours at 4°C. Immunoprecipitated beads were then harvested by centrifugation at 3500g for 1 minute at 4°C. For the kinase reaction, immunoprecipitated beads were washed twice with ice-cold washing buffer solution, 3 times with kinase assay solution containing 50 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid), pH 7.5, 0.1 mM EDTA, and 0.015% Brij35, and then resuspended in 10 μL kinase assay buffer containing 0.1 μg/mL bovine serum albumin (BSA) and 0.2% β-mercaptoethanol. The beads suspension was mixed with 10 μL kinase assay buffer containing 2.5 μg Raytide EL protein kinase substrate or Raytide negative control substrate (Oncogene Research Products, Cambridge, MA). The kinase reaction was initiated by the addition of 10 μL of an adenosine triphosphate (ATP) mixture containing 0.15 mM ATP, 30 mM MgCl2, and 200 μCi/mL (7.4 MBq/mL) [γ-32P] ATP in kinase assay buffer. After incubation at 30°C for 30 minutes, the reaction was stopped by the addition of 120 μL 10% phosphoric acid. Immunoprecipitated beads were removed by centrifugation at 15 000 rpm for 1 minute, and 120 μL supernatant was applied onto a square (2 × 2 cm) of P81 paper. The paper was extensively washed with 0.5% phosphoric acid.
acid, and then relative quantity of $[^{32}P]$ incorporated into phosphorylated protein kinase substrates was measured by liquid scintillation counter (LSC-740; Aloka, Tokyo, Japan).

For quantification of Lyn, part of the immunoprecipitated beads was resuspended in 10 μL sample buffer solution and heated at 100°C for 5 minutes for denaturation. Denatured samples were loaded onto 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The monoclonal antibody mouse anti-Lyn (Santa Cruz Biotechnology) was used for detection of Lyn. Polyclonal antibody reactive against the primary antibody conjugated to horseradish peroxidase was used as a secondary reagent.

## Results

### Inhibition of MS-5–induced proliferation and differentiation by NU-L\textsubscript{PAN}

The ability of the mouse bone marrow stroma cell line MS-5 to induce growth and differentiation of human hematopoietic cells has been reported elsewhere.\textsuperscript{21} MS-5 supported UCB CD34$^+$ cell proliferation 11.9-fold during 2 weeks of culture. Culture gave rise to mixed lineages of myeloid and erythroid cell populations. The stimulation of CD34$^+$ cells with NU-L\textsubscript{PAN} reduced the proliferation to 1.92-fold (Figure 1A). NU-L\textsubscript{PAN} also inhibited MS-5–induced erythroid differentiation of CD34$^+$ cells (Figure 1B). Moreover, CFU-GEMMs and BFU-Es were almost completely absent in the presence of NU-L\textsubscript{PAN} (Figure 1C).

### Inhibition of EPO-induced proliferation and erythroid differentiation by NU-L\textsubscript{PAN}

SCF, IL-3, G-CSF, EPO, and TPO all independently induced the proliferation of CD34$^+$ cells (Figure 2). GM-CSF was also tested; however, no significant induction of CD34$^+$ cell proliferation was observed (Figure 2C). NU-L\textsubscript{PAN} showed a clear inhibition of EPO-induced proliferation, and the number of cells was reduced from $1.23 \times 10^6$ to $5.20 \times 10^4$ cells/mL after 11 days of culture in the presence of NU-L\textsubscript{PAN} (Figure 2E). NU-L\textsubscript{PAN} slightly affected the proliferation of cells stimulated with SCF, G-CSF, and TPO (Figure 2A,D,F). However, NU-L\textsubscript{PAN} did not show any effect on IL-3–induced proliferation (Figure 2B). In addition, NU-L\textsubscript{PAN} clearly inhibited EPO-induced erythroid differentiation of CD34$^+$ cells (Figure 3). Although clear erythroid proliferation and differentiation were observed in the presence of EPO without NU-L\textsubscript{PAN} for 8 to 11 days, the majority of cells in culture in the presence of NU-L\textsubscript{PAN} showed immature erythroid morphology even after 11 days in culture (Figure 3). The cells stimulated with SCF, IL-3, GM-CSF, G-CSF, and TPO, with or without the addition of NU-L\textsubscript{PAN}, were morphologically similar (data not shown).

### Characterization of NU-L\textsubscript{PAN}–stimulated cells

In the presence of EPO, the expression of CD34 and glycophorin A was down-regulated and up-regulated, respectively. NU-L\textsubscript{PAN} slightly prolonged these effects (Figure 4A-B). However, more than 90% of the cells were CD34$^+$, glycophorin A$^+$ after 8 days in cultures both with and without NU-L\textsubscript{PAN}, and hemoglobin synthesis in the cells cultured with or without the addition of NU-L\textsubscript{PAN} was similar (Figure 4). Based on the reports by Okumura et al.\textsuperscript{23} and Nakahata et al.\textsuperscript{25} morphologic and immunologic characteristics indicated that the NU-L\textsubscript{PAN}–stimulated cells were compatible with basophilic erythroblasts.
CD45 inhibits erythroid differentiation

In addition to the above effect of NU-L\textsubscript{PAN} on erythroid differentiation, NU-L\textsubscript{PAN} also affected the proliferation of immature erythroid progenitor cells. The number of BFU-Es after EPO stimulation was decreased after addition of NU-L\textsubscript{PAN}; however, NU-L\textsubscript{PAN} did not affect CFU-GEMMs and CFU-GMs (Figure 5).

Inhibition of EPO-induced phosphorylation of Lyn by NU-L\textsubscript{PAN}

When erythroblasts obtained after 4 days culture of CD34\textsuperscript{+} cells in the presence of EPO were restimulated with EPO, the cells showed phosphorylation of JAK2 and Lyn (Figure 6). Preincubation of the cells with NU-L\textsubscript{PAN} for 18 hours prior to EPO stimulation revealed a reduced level of phosphorylated Lyn but not that of JAK2 (Figure 6). Corresponding with the reduced level of phosphorylation, the kinase activity of Lyn was also inhibited by the NU-L\textsubscript{PAN} treatment (Figure 7).

Discussion

CD45 is known as a membrane-bound tyrosine phosphatase that regulates T- and B-cell activation through dephosphorylation of Src family kinases.\textsuperscript{1-5} CD45 is expressed on most hematopoietic cells except for mature red blood cells and platelets. However, the role of CD45 in hematopoietic differentiation has only been partially addressed.\textsuperscript{1,2,14} Irie-Sasaki et al\textsuperscript{13} and Penninger et al\textsuperscript{14} reported on a novel function of CD45 as a phosphatase of JAKs. They reported that CD45 down-regulated the proliferation and differentiation of hematopoietic cells through dephosphorylation of JAKs. JAKs have been known to play an indispensable role in the signaling pathways of most hematopoietic cytokine receptors including those of EPO and IL-3 receptors.\textsuperscript{15,16}

To confirm the role of CD45 in hematopoietic differentiation, we investigated the effects of the anti-CD45 antibody, NU-L\textsubscript{PAN}, using UCB CD34\textsuperscript{+} cells as a target. NU-L\textsubscript{PAN} was found to strongly inhibit the proliferation and differentiation of CD34\textsuperscript{+} cells (Figure 1). As a possible mechanism, we first considered dephosphorylation of JAKs induced by CD45 signaling. However, NU-L\textsubscript{PAN} clearly hampered EPO-induced proliferation and erythroid differentiation of CD34\textsuperscript{+} cells but not that of IL-3–induced proliferation of the cells (Figures 2-5). In fact, NU-L\textsubscript{PAN} did not inhibit EPO-induced phosphorylation of JAK2 (Figure 6). Our experiments showed that CD45 signaling inhibited erythropoiesis in a manner rather different from the mechanism of inhibition of JAKs.

On the other hand, preincubation of cells with NU-L\textsubscript{PAN} for 18 hours prior to EPO stimulation revealed a reduced level of phosphorylated Lyn (Figure 6). The reduced level of phosphorylation correlates with the reduced kinase activity of Lyn (Figure 7). CD45 is known as a phosphatase that acts on Src family kinases including Lyn, and either positively or negatively controls the said kinase activities through the dephosphorylation of tyrosine residues at positive or negative regulatory sites as described in the “Introduction.” Therefore, we concluded that CD45 is activated by the binding with NU-L\textsubscript{PAN} and continuously dephosphorylates the positive regulatory site on Lyn to inhibit its kinase activity. Lyn is known to associate with various cytokine receptors such as those for EPO and IL-3.\textsuperscript{17,20,26,27} Although important roles for Lyn in

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EPO-induced erythroid differentiation have been described, the role for Lyn in IL-3–induced proliferation and differentiation of hematopoietic cells is still unknown. 16–20 Tilbrook et al 18 reported that an immature erythroid cell line J2E has the ability to differentiate into mature erythrocytes after EPO stimulation. A mutant clone of J2E, J2E-NR, was originally isolated due to its inability to differentiate in response to EPO. J2E-NR showed markedly reduced expression of Lyn, and transfection of J2E-NR with a retroviral vector carrying Lyn restored its responsive-ness to EPO. Dominant-negative forms of Lyn transfected into J2E showed erythroid differentiation arrest. 19 Other researchers reported an association between Lyn and the EPO receptor and a role for Lyn in the proliferative effects of EPO. 21 Nevertheless, it is assumed that the effects observed in this study are due to CD45 itself and are not artifactual due to other nonspecific effects of NU-LPAN antibody. However, Broxmeyer et al 22 reported results discordant from our experiments showing that certain anti-CD45 antibodies inhibit the proliferation of myeloid progenitors but not that of erythroid progenitors. Thus, CD45 signaling varies between different target cells and antigenic epitopes. We show here that NU-LPAN inhibits erythroid differentiation of UCB CD34+ cells similar to NU-LPAN (data not shown). This result shows that the ability to produce cells of all hematopoietic lineages. This capacity with the sugar residue of CD45; however, there remain candidates for the natural ligand of CD45 due to their binding specificity to CD45RB or not. However, EPO acts as an excellent erythroid potentiator in vitro as well as in vivo. We have demonstrated here CD45 plays a pivotal role in the negative regulation of erythroid proliferation and differentiation in vitro. NU-LPAN could be a useful material for further investigation into the negative regulatory mechanisms in erythropoiesis.

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References


