Tumor Necrosis Factor (TNF) Is a Physiologic Regulator of Hematopoietic Progenitor Cells: Increase of Early Hematopoietic Progenitor Cells in TNF Receptor p55-Deficient Mice In Vivo and Potent Inhibition of Progenitor Cell Proliferation by TNFα In Vitro


Murine bone marrow cells with lineage phenotypes (Lin-) Sca-1-'c-kit' and Lin 'Sca-1'-'c-kit' cells represent primitive hematopoietic stem cells (HSCs) and committed hematopoietic progenitor cells, respectively. The number of Lin 'Sca-1'-'c-kit' HSCs in bone marrow was significantly increased in tumor necrosis factor (TNF) receptor p55-deficient (TNF-R55-/-) mice compared with the TNF-R55+/+ wild-type mice without a marked change in bone marrow cellularity. In both the methylcellulose culture and a single-cell proliferation assay, mouse TNFα (mTNFα) inhibited in vitro the proliferation of wild-type mouse-derived Lin 'Sca-1'-'c-kit' cells in response to a combination of multiple growth factors. The same is true for that of Lin 'Sca-1'-'c-kit' cells stimulated with granulocyte colony-stimulating factor (G-CSF) plus stem cell factor (SCF). Moreover, mTNFα significantly arrested the entry into S-phase from G1/G0 phase of Lin 'Sca-1'-'c-kit' cells stimulated with multiple growth factors and Lin 'Sca-1'-'c-kit' cells stimulated with G-CSF plus SCF. In contrast, mTNFα failed to affect the growth and cell cycle progression of Lin 'Sca-1'-'c-kit' cells and Lin 'Sca-1'-'c-kit' cells that were obtained from TNF-R55-deficient mice. These data suggest that TNF may be an important physiologic regulator of hematopoiesis and that TNF-R55 may be essentially involved in TNF-mediated inhibition of the growth of both primitive stem and more committed progenitor cells.

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centration as follows: IL-3, 2 ng/mL; SCF, 2 ng/mL; GM-CSF, 2 ng/mL; G-CSF, 2 ng/mL; and IL-6, 100 U/mL. An anti-c-kit antibody (ACK-2) was kindly provided by Dr T. Sudo (Toray, Kanazawa, Japan). Monoclonal antibodies (MoAbs) used for the immunostaining as follows: IL-3, 2 ng/mL; SCF, 2 ng/mL; GM-CSF, 2 ng/mL, G-CSF, 2 ng/mL; and IL-6, 100 U/mL. An anti-c-kit antibody (NK-55) was used for the immunostaining.

Isolation of Lin-"Sca-1"-c-kit" HSCs and Lin-"Sca-1"-c-kit" HPCs. TNF-R55-/- mice were generated as previously described and maintained by mating with C57BL/6 mice under pathogen-free conditions in the Animal Research Center of Kanazawa University (Kanazawa, Japan). Bone marrow cells were obtained by aspirating femurs of 11-week-old TNF-R55-/- mice (denoted as wild-type thereafter) or homozygous TNF-R55-/- mice. Mononuclear cells (MNCs) were separated by centrifugation on a Histopaque-1077 (Sigma Chemical Co., St. Louis, MO). The cells were suspended in Iscove's modified Dulbecco's medium (IMDM; GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS; ICN Biomedicals Japan Co Ltd, Osaka, Japan) and washed twice, and then fixed in 70% ethanol for more than 24 hours. After being denatured with 2 N HCl at room temperature for 30 minutes and subsequently washed twice with 50% Tween-20:phosphate-buffered saline (PBS), the cells were stained with a concentration of 5.0 μg/mL of mouse anti-BrdU MoAb at 4°C overnight followed by the incubation with FITC-conjugated goat-IgG(Fab')2 antimmunoglobulin (Caltag, Camarillo, CA) and streptavidin-conjugated phycoerythrin. The total numbers of nucleated cells in wild-type and in Lin-Sca-1-c-kit+ HSCs and Lin-Sca-1-c-kit+ HPCs in bone marrow from TNF-R55-/- mice were similar, ie, 2.8 X 10^6 and 2.6 X 10^6, respectively. Thereafter, the subpopulations of isolated Lin- cells showed that the proportion of Lin-Sca-1+c-kit+ cells and, to a lesser degree, to a lesser degree, the proportion of Lin-"Sca-1"-c-kit" cells were further purified by using two cycles of cell sorting. The contamination of other types of cells in each of these populations was consistently less than 1% as shown by immunofluorescence analysis.

Methylcellosolve culture of progenitor cells. Colony formation by murine bone marrow stem and progenitor cells was evaluated according to the method of Stanley et al., with some modifications. Briefly, either Lin-"Sca-1"-c-kit" or Lin-"Sca-1"-c-kit" cells were plated in 1 mL of IMDM containing 0.9% methylcellulose (Shinetsu Chemical Co, Tokyo, Japan), and 20% heat-inactivated FCS, 1% bovine serum albumin (BSA, Sigma Chemical). Lin-"Sca-1"-c-kit" HSCs were stimulated with the combination of IL-3 plus SCF or IL-3 plus SCF. mTNFa was added to the cultures for 72 hours, the number of viable cells was counted using trypan blue staining. Cell cycle analysis was performed as previously described by Laiho et al., with a slight modification. Briefly, the cells were labeled with 50 μmol/L 5-bromo-2'-deoxy uridine (5-BrdU; Sigma Chemical) for 4 hours before the indicated time points and were fixed in 70% ethanol for more than 24 hours. After being denatured with 2 N HCl at room temperature for 20 minutes and subsequently washed twice with 0.5% Tween-20:phosphate-buffered saline (PBS), the cells were stained with a concentration of 5.0 μg/mL of mouse anti-BrdU MoAb at 4°C overnight followed by the incubation with FITC-conjugated goat-IgG(Fab')2 antimouse IgG at room temperature for 1 hour. After washing twice, the cells were treated with 1 mg/mL of RNAase at room temperature for 30 minutes and stained with 20 μg/mL of propidium iodide (Sigma Chemical) in PBS for 30 minutes. Cell cycles were analyzed based on BrdU incorporation and DNA content using the EPICS Elite according to the manufacturer's instructions.

RESULTS

Increases in the numbers of Lin-"Sca-1"-c-kit" HSCs in TNF-R55-/- mice. Because several lines of observations suggest potential roles of TNFα in hematopoiesis, we first determined the number of Lin-"Sca-1"-c-kit" HSCs and Lin-"Sca-1"-c-kit" HPCs in bone marrow from TNF-R55-/- as well as wild-type mice. We pooled bone marrow nucleated cells that were collected from two femora and two tibias. The total numbers of nucleated cells in wild-type and in TNF-R55-/- mice were similar, ie, 2.8 ± 0.71 X 10^7 and 2.6 ± 0.62 X 10^7 cells (n = 4), respectively. Compared with the bone marrow from wild-type mice, there was no change in cellularity and cellular architecture in the bone marrow of TNF-R55-/- mice (data not shown). However, immunofluorescence analysis showed a 1.6-fold increase over normal (21.6% ± 3.2%; n = 4) in the number of Lin-"Sca-1"-c-kit" cells in TNF-R55-/- mice (33.8% ± 3.0%; n = 4). Further analysis of subpopulations of isolated Lin-"Sca-1"-c-kit" cells showed that the percentage of Lin-"Sca-1"-c-kit" cells was increased in TNF-R55-/- mice compared with wild-type mice (Fig 1). Moreover, the proportion of Lin-"Sca-1"-c-kit" cells and, to a lesser degree, to a lesser degree, the proportion of Lin-"Sca-1"-c-kit" cells were significantly increased in the mutant mice (Fig 1). Collectively, these observations show that TNFα may negatively regulate the development of early HPCs in vivo by interacting with TNF-R55.

Growth inhibition of Lin-"Sca-1"-c-kit" HSCs and Lin-"Sca-1"-c-kit" HPCs by TNFαs in a methylcellulose culture. We next examined the in vitro effects of TNFα on the proliferative capacities of highly purified HSCs and HPCs obtained
Fig 1. Expression of c-kit and Sca-1 on Lin- bone marrow progenitor cells derived from (A) wild-type and (B) TNF-R55" mice. After isolating Lin- progenitor cells from bone marrow MNCs, the cells were analyzed using a two-color immunofluorescence as described in Materials and Methods. The figures in each quadrant represent the percentage of cells.

Fig 2. Effects of mouse and human TNFα on growth factor-induced proliferation of Lin-Sca-1"c-kit" HSCs obtained from wild-type mice (A) or TNF-R55" mice (B). The highly purified Lin-Sca-1"c-kit" HSCs were plated in a methylcellulose medium containing the combination of multiple growth factors with and without mTNFα of increasing doses, as indicated. The numbers of the large-sized colonies (>0.5 mm) and small-sized colonies (<0.5 mm) were determined for the growth of high-proliferative potential CFCs and low-proliferative potential CFCs, respectively. The data are representative from three independent experiments with quadruplicate determinations. Error bars and asterisks indicate SD and significant differences by one-way analysis of variance (ANOVA; P < .05 v growth factors alone), respectively.
tion of both large- and small-sized colonies induced by G-CSF plus SCF (Fig 3A). However, when stimulated with IL-3 plus SCF, Lin'Sca-1'c-kit' HSCs but not Lin'Sca-1'c-kit' HPCs could be inhibited with mTNFα to form large-

sized colonies (Fig 4A). The same is true for the combination of multiple growth factors (data not shown). These data suggest that TNFα can inhibit the proliferation of primitive Lin'Sca-1'c-kit' HSCs regardless of the growth factor supplemented. However, the response of Lin'Sca-1'c-kit' HPCs to TNFα may change, depending on the growth factors used.

The failure of mTNFα to inhibit the growth of CFC from either subpopulation of stem and progenitor cells obtained from TNF-R55+/− mice. To assess the role of TNF-R55 in TNFα-mediated inhibition of CFC growth, we next examined the effects of mTNFα on the proliferation of Lin'Sca-
1 c-kit+ HSCs and Lin−Sca-1 c-kit+ HPCs from wild-type mice. mTNFa did not affect the growth of either primitive Lin−Sca-1 c-kit+ cells stimulated with multiple growth factors (Fig 2B), that of Lin−Sca-1 c-kit+ cells incubated with G-CSF plus SCF (Fig 3B), or those of the two subpopulations stimulated with IL-3 plus SCF even at the highest concentration used (Fig 4B). Collectively, these results suggest that mTNFa exerts its inhibitory effects on the proliferation of HSCs and HPCs mainly by interacting with TNF-R55.

**Growth inhibition of Lin−Sca-1 c-kit+ HSCs from wild-type mice by TNFa in a single-cell proliferation assay.** A single-cell proliferation assay was performed to exclude the possibility of indirect effects of TNFa. mTNFa inhibited the proliferation of IL-3 plus SCF-induced Lin−Sca-1 c-kit+ HSCs (Table 1). In addition, it inhibited the growth of highly proliferative cells from Lin−Sca-1 c-kit+ HSCs in response to multiple growth factors, whereas low-proliferative potential cells were less affected (Table 1). Moreover, TNFa also inhibited the growth of Lin−Sca-1 c-kit+ cells stimulated with G-CSF plus SCF (data not shown). Thus, TNFa directly inhibited the growth of primitive progenitor cells, particularly those with high proliferative potentials.

**Cell cycle arrest of Lin−Sca-1 c-kit+ and Lin−Sca-1 c-kit+ cells by mTNFa.** Hematopoietic growth factors regulate the proliferation of stem and progenitor cells by inducing the entry of G0/G1-phase cells into S phase or maintaining the survival of G0/G1-phase cells in dormancy. Hence, we finally examined the effects of mTNFa on the cell cycle of wild-type Lin−Sca-1 c-kit+ cells incubated with multiple growth factors and of Lin−Sca-1 c-kit+ cells incubated with G-CSF plus SCF in a suspension culture. mTNFa decreased the numbers of both subpopulations at 72 hours (Table 2). The cell viability in each group was more than 95% as determined by trypan blue staining. In agreement with the growth inhibition, mTNFa markedly arrested Lin−Sca-1 c-kit+ HSCs and Lin−Sca-1 c-kit+ HPCs in G0/G1-phase and significantly decreased the percentage of S-phase cells (Table 2). However, mTNFa affected neither the numbers of viable cells nor the cell-cycle transition of TNF-R55+ mice-derived HSCs and HPCs (Table 2). These results suggest that signalling of TNF through TNF-R55 is antiproliferative for hematopoietic stem and progenitor cells by blocking their cell cycle.

**DISCUSSION**

Previous in vitro observations have established TNFa as a bifunctional regulator of hematopoiesis. A single-dose injection of TNFa stimulates the growth of immature murine myeloid progenitors, whereas daily injection of TNFa induces a slight decrease of early myeloid progenitors with neutrophilia and lymphopenia. In contrast, no remarkable changes in bone marrow cellularity have been reported in TNFa transgenic mice. However, there has not been direct evidence for a physiologic role of TNFa in growth regulation of early hematopoietic stem and progenitor cells in vivo.

Gene targeting has become a powerful tool to show novel and unexpected roles of the protein encoded by a targeted gene. Because TNFβ binds to the same receptors as TNFa, the effects of TNFa gene disruption might be compensated by TNFβ. Hence, we examined bone marrow stem and progenitor cells from TNF-R55 gene knockout mice. The results showed that the absolute number of primitive Lin−Sca-1 c-kit+ HSCs and, to a lesser degree, that of Lin−Sca-1 c-kit+ HPCs were significantly increased in the bone marrow of TNF-R55−/− mice. Moreover, TNF-R55+−/− deficient Lin−Sca-1 c-kit+ HSCs gave rise to a twofold greater number of large-sized colonies in a methylcellulose culture (Fig 2B) than those from wild-type HSCs (Fig 2A). Thus, TNFa may negatively regulate the growth of primitive Lin−Sca-1 c-kit+ HSCs even in vivo by interacting essentially with TNF-R55 in the course of normal hematopoiesis.

Previously, agonistic antibodies or TNFa mutants that selectively agonize one of the TNF-Rs have been used to dissect the role of TNF-Rs in regulating the growth of human HPCs in vitro. TNFa stimulates in vitro GM-CSF−/− or IL-3− induced colony formation and inhibits G-CSF−/− or SCF− induced proliferation of human CD34+ HPCs through TNF-R55. In contrast, both TNF receptors are required to inhibit the growth of primitive human CD34+ hematopoietic progenitor cells stimulated with multiple cytokines. In our systems, mTNFa failed to affect in vitro the proliferation and cell cycle progression of TNF-R55−/− deficient Lin−Sca-1 c-kit+ HSCs and Lin−Sca-1 c-kit+ HPCs. Thus, TNF-R55 is the essential receptor for mediating an inhibitory signal in Lin−Sca-1 c-kit+ HSCs in our experimental systems. In contrast to our observations, an agonistic antibody to TNF-R75, but not to TNF-R55, can inhibit the growth of murine primitive Lin−Sca-1+ progenitor cells. The different experimental systems used in the study, other possible explanations for the discrepancy might be the use of different types of primitive progenitor cells (Lin−Sca−1+ in their experiments versus Lin−Sca-1+ in ours). Lin−Sca-1+ cells have been shown to be heterogeneous stem and progenitor cells and can be separated into several sub-types.
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Table 2. The Effect of mTNFα on the Cell Cycle Transition of Lin-"Sca-1"-c-kit"HSCs and Lin-"Sca-1"-c-kit"HPCs

<table>
<thead>
<tr>
<th></th>
<th>Cell No. (x10⁵/mL)</th>
<th>G₀/G₁ (%)</th>
<th>S (%)</th>
<th>G₂/M (%)</th>
<th>Cell No. (x10⁵/mL)</th>
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<td>16</td>
<td>78</td>
<td>2.3</td>
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<td>TNF-R55&quot;-&quot;</td>
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<td>Lin-&quot;Sca-1&quot;-c-kit&quot; HSCs</td>
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<td>18</td>
<td>78</td>
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<td>38</td>
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<td>13</td>
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Lin-"Sca-1"-c-kit"HSCs and Lin-"Sca-1"-c-kit"HPCs were obtained from either wild-type or TNF-R55"-" mice and were plated in a suspension culture in IMDM with 20% FCS supplemented with the combination of multiple growth factors and of G-CSF plus SCF, respectively. mTNFα (final concentration, 300 U/mL) was added into the cultures and incubated at 37°C and 5% CO2 in air for 72 hours. The number of recovered cells was expressed as the cell number x 10⁵/mL, whereas the proportion of cell cycle was expressed as the percentage of each cell cycle after the analysis by use of 5-BrdU immunostaining combined with propidium iodide, as described in Materials and Methods. The data shown here are representative of three independent experiments.

sets.40-42 Lin-"Sca-1"-c-kit" HSCs may represent one of the subsets on which TNFα may exert its negative regulatory effects essentially through TNF-R55 pathway and are therefore increased in TNF-R55"-" mice.

TNFα exhibits its bifunctional regulatory effects on hematopoiesis, depending on the progenitor cells used and growth factors supplemented.1,3,12 Several groups reported that TNFα can induce production of other hematopoietic growth factors, thereby stimulating indirectly the growth of bone marrow stem and progenitor cells.9,43,45 In contrast, mTNFα directly inhibits the proliferation of enriched hematopoietic progenitors as determined by a single-cell proliferation assay.10-12 Downregulation of receptors for growth factors such as G-SCF, IL-3, GM-CSF, and SCF receptors has been documented on low-density bone marrow cells or heterogeneous progenitor cells incubated with TNFα.9,10,12,46 However, SCF receptors on human CD34⁺ progenitor cells are reportedly downregulated by TNFα only marginally, whereas TNFα markedly inhibits proliferation of these progenitor cells induced by SCF or combined with other growth factors.10,11 TNFα directly potentiates the proliferation of IL-3- or GM-CSF-induced human CD34⁺ progenitor cells,5,6,8 whereas IL-3 or GM-CSF receptors are downregulated on low-density bone marrow cells.9,11 Thus, downregulation of growth factor receptors by TNFα cannot explain the mechanisms of the effects of TNFα on hematopoiesis.

Inhibitory effects of TNFα on cell cycles have been observed on committed CD15⁺ myeloid progenitors.5 We show here that TNFα could prevent the transition of more primitive HSCs or committed HPCs from G₀/G₁ to S phase without significantly affecting the cell viability. The inhibitory effects of TNFα on cell cycle transition of bone marrow stem and progenitor cells may account for protective activities of TNFα on hematopoietic progenitor cells from cytotoxicity of cell cycle-specific cytotoxic chemical drugs.47 In addition, the induction of cell cycle arrest by TNFα may be responsible for the antiproliferative effects on HSCs or HPCs. We observed that the effects can be reversed on the removal of TNFα, particularly on Lin-"Sca-1"-c-kit" HSCs (data not shown), consistent with the previous report that inhibitory effects of TNFα on early hematopoietic progenitors are reversible.52 Hence, TNFα prevent cell cycle transition without causing irreversible events such as apoptosis or necrosis, although the contribution of these phenomena cannot be excluded completely. Large numbers of highly purified stem and progenitor cells are required for biochemical and molecular biologic analyses on TNFα-induced cell cycle arrest.

Hematopoietic growth factors can regulate the cell cycles of bone marrow progenitor and stem cells in differential ways. IL-3 stimulates the proliferation of murine multipotential progenitors in active cell division phase and maintains the survival of dormant progenitor cells.35,36,48,49 In contrast, G-CSF, SCF, IL-6, and IL-11 shorten the dormancy period of murine progenitors.50-52 In addition, SCF and GM-CSF are also able to maintain the survival of dormant progenitor cells. Thus, the effect of TNFα on the growth of hematopoietic stem and progenitor cells may change, depending in which phase of the cell cycle they are.

Endogenous production of TNFα has been observed in various leukemia cells, such as acute myeloid leukemia cells,44,53 chronic B-lymphoblastic leukemia cells,46 hairy cell leukemia cells,57 and residual host blood cells from allogeneic bone marrow transplantation patients.48 Our results suggested that TNFα can suppress the growth of early hematopoietic progenitor cells in vivo and in vitro. Thus, blocking of these activities and/or production of TNFα might be clinically useful in treating the hematopoietic failure in these diseases.

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Tumor necrosis factor (TNF) is a physiologic regulator of hematopoietic progenitor cells: increase of early hematopoietic progenitor cells in TNF receptor p55-deficient mice in vivo and potent inhibition of progenitor cell proliferation by TNF alpha in vitro

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