Expression of a Functional c-kit Receptor on a Subset of Natural Killer Cells

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Summary

Natural killer (NK) cells are large granular lymphocytes thought to be important in the host's early immune response to viral infection and malignant transformation. NK cells proliferate and display enhanced cytotoxic activity in response to the T cell growth factor, interleukin 2 (IL-2). Stem cell factor or steel factor (SF) is the ligand for the c-kit receptor, and when combined with other hematopoietic growth factors, SF synergistically promotes the proliferation and differentiation of bone marrow stem cells. In the present study we show the c-kit receptor to be uniquely expressed on a subset of resting human NK cells (CD56b~s ht) which constitutively expresses both the high affinity IL-2 receptor (IL-2R) and the intermediate affinity IL-2R. Other lymphocyte populations, including CD56 dim NK cells, did not appear to express the c-kit receptor. Within the CD56b~s ht NK cell subset, SF alone had no obvious effect on proliferation or cytotoxic activity. SF was shown to significantly augment the proliferative effect of IL-2, and caused a marked shift in the dose–response curve at IL-2 concentrations that selectively saturate the high affinity IL-2R. The potentiating effect of SF on NK cell proliferation was dependent on IL-2 binding to the high affinity IL-2R, and was blocked by a monoclonal antibody directed against the c-kit receptor. SF did not enhance proliferation at higher IL-2 concentrations that saturate the intermediate affinity IL-2R, nor did SF enhance IL-2–induced cytotoxic activity. Together, these data indicate that SF and IL-2 act synergistically to directly augment the proliferative capacity of a unique human NK cell subset constitutively expressing the high affinity IL-2R and the c-kit receptor. The implications of these findings on NK cell development and the host's early immune response to pathogen invasion are discussed.

NK cells are a distinct population of large granular lymphocytes that appear to have an important role in the host's early immune response to viral infection and malignant transformation. NK cells show a proliferative and enhanced cytotoxic response to IL-2, a lymphocytotropic hormone produced by activated T cells that is essential in normal immune responses (for a review see reference 1). Indeed, unlike the vast majority of other lymphocyte populations found in resting human peripheral blood, most NK cells constitutively express one or two functional isoforms of the human IL-2R. 90% of NK cells can be identified by their low density expression of the CD56 antigen (CD56dim) and express an intermediate affinity IL-2R, now known to be composed of at least two subunits, IL-2β and IL-2γ. A minor subset of NK cells, identified by its high density expression of the CD56 antigen (CD56bright), expresses both the intermediate affinity IL-2R and the high affinity IL-2R, composed of three subunits that are noncovalently linked to form a heterotrimer (IL-2Rαβγ) (2–5). Saturation of the high affinity IL-2R expressed on the CD56bright NK population with low concentrations of IL-2 (i.e., 1–100 pM) results in a profound proliferative response with only modest enhancement of cytotoxic activity. The intermediate affinity IL-2R, when saturated by higher concentrations of IL-2 (i.e., 1–10 nM), significantly enhances cytotoxic activity in both the CD56bright and CD56dim NK cell populations, yet does not result in any enhancement of proliferation (2). Thus, despite both functional IL-2R isoforms being constitutively expressed on the CD56bright NK cells and both isoforms possessing the subunits important for signal transduction and internalization (5), significant proliferation only occurs via the heteromeric high affinity IL-2Rαβγ.
The protoonogene c-kit encodes a transmembrane tyrosine kinase receptor that belongs to a superfamily that includes the receptors for insulin, platelet-derived growth factor, epidermal growth factor, and CSF-1 (6). Within the hematopoietic system the c-kit receptor is expressed on normal bone marrow cells at various stages of maturation (7). Steel factor (SF) or stem cell factor is a ligand for the c-kit receptor (8, 9), and together, c-kit receptor and SF are considered to play a major role in the regulation of human hematopoiesis. Whereas SF alone has only a modest effect on stem cell proliferation (10), SF has been observed to exhibit potent synergistic activity with virtually all of the known hematopoietic growth factors, including erythropoietin (Epo), IL-3, GM-CSF, G-CSF, and IL-6, resulting in increased colony formation of both primitive and lineage-specific hematopoietic progenitor cell populations (11, 12). These hematopoietic growth factors specifically bind to their respective receptors which all belong to the hematopoietin receptor superfamily that also includes IL-2Rβ and IL-2Rγ (5, 13). Because SF has been shown to augment the proliferative effects mediated via the hematopoietin receptors constitutively expressed on hematopoietic stem cells, we hypothesized that SF may also potentiate functional responses of IL-2 in human NK cells that constitutively express IL-2R. In the present study, we demonstrate that the CD56bright NK cell appears unique among resting human lymphocytes in its constitutive expression of the c-kit receptor, and that has performed a functional characterization of the interactions between SF and IL-2 on this lymphocyte population.

Materials and Methods

mAbs. Nonreactive mouse immunoglobulin (M1g) was purchased from Sigma Immunochemicals (St. Louis, MO). NKH1 (anti-CD56)-PE was from Coulter Immunology (Hialeah, FL). Leu16 (anti-CD20)-PE, and Leu4 (anti-CD5)-PE, were from Becton Dickinson & Co. (San Jose, CA). YB5.B8 is the anti-c-kit receptor mAb (14). Clone D3.3.3 is a nonreactive isotype control. Purified anti Tac (anti-CD25) was kindly provided by Dr. Kendall Smith (Cornell Medical School, New York), and purified mAb 2T8-SH7 was kindly provided by Dr. Stuart Schlossman (Dana Farber Cancer Institute, Boston, MA). 3F5B11 is a IgG1 murine mAb developed in our laboratory and is reactive with monomorphic HLA-DR. FITC-conjugated goat anti-mouse IgG was from Tago, Inc. (Burlingame, CA).

Immunofluorescence Analysis of Lymphocyte Subsets for c-kit Receptor Expression. PBL were analyzed for simultaneous two-color immunofluorescent expression of the c-kit receptor and either CD56bright and CD56dim (NK cells), CD20 (B cells), or CD3 (T cells). Fresh peripheral blood was obtained from healthy donors for isolation of PBMC with Ficoll-Hypaque separation, and adhered for 1 h at 37°C. 10 6 nonadherent PBMC were then incubated on ice with either anti-c-kit receptor mAb or with a nonreactive isotype control mAb, washed once, incubated with goat anti-mouse-FITC, washed twice, and incubated with directly conjugated mAb CD56-PE, CD20-PE, CD3-PE, or a nonreactive M1g-PE control mAb. Background fluorescence was determined on cells stained with the nonreactive IgG1 isotype control plus indirect FITC and the directly conjugated nonreactive PE-control. All of the 10,000 cells were then analyzed in each sample using the lymphocyte gate on a FACScan® and results were displayed as an orthographic projection plotting log green vs log red fluorescence using the Lysis 1® software program (Becton Dickinson & Co.). CD56bright and CD56dim cells were separated based on fluorescence intensity of CD56 (2). Because the initial analysis of 10,000 PBL collected <200 CD56bright cells, the FL-2 amplifier gain of the FACScan® was adjusted to trigger selectively on the CD56bright cells while analyzing ~10 5 nonadherent PBL which were simultaneously stained with anti-CD56-PE and either control mAb-FITC or anti-c-kit receptor-FITC. 3,000 CD56bright cells were collected and analyzed on six different individuals.

CD56bright NK Cell Isolation. Fresh nonadherent PBMC were depleted of T cells, monocytes, B cells, and HLA-DR+ progenitor cells by incubating PBL in anti-CD4 (1:1000), anti-CD5 (1:400), and anti-HLA-DR (1:100) sterile murine ascites, washing twice, and then incubating with goat anti-mouse mAb coupled to immunomagnetic beads (Advanced Magnetics, Inc., Cambridge, MA). Cells were next adhered to a MaxSep Magnetic Cell Separator (Baxter HealthCare Corp., Deerfield, IL) for 10 min. Nonadherent cells were then labeled with directly conjugated mAbs and sorted for CD56bright, CD56dim, CD56bright c-kit receptor-positive (CD56bright c-kit+), or CD56bright c-kit- cells on a FACStar Plus® cell sorter (Becton Dickinson & Co.).

Proliferation Assays. Sorted CD56bright and CD56dim NK cells were plated in U-bottomed wells at a concentration of 2.0 10 6 cells/well in RPMI 1640 with 10% human AB serum (Gibco Laboratories, Grand Island, NY) in the absence or presence of varying concentrations of recombinant human IL-2 (sp act 1.5 10 7 U/ml; Hoffmann-La Roche, Inc., Nutley, NJ) and varying concentrations of recombinant SF (~36,000 M U; 10 8 U/mg protein; Immunex, Seattle, WA). Cells were incubated at 37°C for 72 or 96 h as indicated. Proliferation was measured by methyl-[H]thymidine incorporation during the last 12 h of incubation. For high affinity IL-2R binding experiments, anti-Tac (anti-CD25) mAb was used as affinity-purified sterile ascites and was added 15 min before the addition of IL-2 (2). For c-kit receptor blocking studies, anti-c-kit receptor mAb (14) was used as affinity-purified sterile ascites and was added 15 min before the addition of SF. Isotype control mAbs, affinity purified in an identical fashion to experimental reagents, were used in blocking studies. Cell number and viability were determined by trypan blue dye exclusion. Cells incubated in "medium only" were in RPMI 1640 supplemented with 10% human AB serum.

Cytotoxicity Assays. Chromium release assays were performed in triplicate as described (2). Sorted populations of CD56bright c-kit+ and CD56dim c-kit- cells were plated in medium alone and mixed immediately with 4 10 5 51Cr-labeled K562 target cells (E/T 10:1), or incubated for 18 h at 37°C in the presence of 10 nM IL-2 (~2,300 U/ml) and then mixed with 4 10 5 51Cr-labeled COLO 205 target cells (E/T 10:1).

ELISA for SF. A sandwich-type ELISA to determine the concentration of SF in RPMI 1640 medium containing 10% human AB serum was kindly performed by L. G. Bennett at Amgen, Inc. (Thousand Oaks, CA) (15).

Statistical Analysis. Results of experimental points obtained from multiple experiments were reported as the mean ± 1 SE. Significance levels were determined by two-sided student's t test analysis.

Results

Selective Expression of the c-kit Receptor on the CD56bright Subset of Human NK Cells in Resting Human PBL. Fresh
PBL were analyzed for the expression of the c-kit receptor in combination with a series of mAbs specific for various lymphocyte populations. Surprisingly, the only lymphocyte population to consistently show constitutive expression of the c-kit receptor was the CD56bright subset of NK cells, which represents <2% of PBL. CD56dim NK cells and CD56neg lymphocytes failed to demonstrate any reactivity with the anti-c-kit receptor mAb which was above background staining (Fig. 1). In an analysis of six different individuals, 45.1 ± 8.6% of CD56bright cells coexpressed c-kit receptor (CD56 bright c-kit+). Other lymphocyte populations, including CD3+ T and CD20+ B cells failed to show any significant expression of the c-kit receptor by flow cytometric analysis. Incubation of NK cells in 1 nM IL-2 did not result in an upregulation of c-kit receptor expression on CD56bright or CD56dim NK cells, and incubation in SF did not appear to upregulate CD25 (IL-2Rα) or IL-2Rβ expression (data not shown).

The CD56bright c-kit+ Cell Demonstrates both NK and LAK Activity. NK cells are best defined functionally by their ability to lyse target cells without deliberate prior sensitization and without restriction by MHC antigens (NK activity). In addition, NK cells demonstrate cytotoxic activity against NK-resistant target cells after exposure to IL-2 (LAK activity) (1). To show that CD56bright c-kit+ cells possessed NK activity and were responsive to IL-2, CD56bright c-kit+ and CD56dim c-kit- cells were sorted by FACS® and plated immediately in a cytotoxicity assay against the NK-sensitive K562 cell line, or incubated overnight in 10 nM IL-2 and then plated in a cytotoxicity assay against the NK-resistant COLO 205 cell line. The results, shown in Fig. 2, demonstrate that CD56bright c-kit+ cell possesses significant NK and LAK activity, although less than the CD56bright c-kit- fraction.

The effect of SF on NK and LAK activity was also evaluated. When resting CD56bright NK cells were cultured in a high (~7 nM or 250 ng/ml) concentration of SF alone, cytotoxicity against K562 tumor cell targets was not significantly increased over that seen in medium alone. Furthermore, the addition of ~7 nM SF to cultures containing low (10 pM or ~2.3 U/ml) or high (1 nM or ~230 U/ml) concentrations of IL-2 did not enhance the LAK activity mediated via the high or intermediate affinity IL-2R, respectively (data not shown).

Effects of SF on IL-2-induced NK Cell Proliferation. Highly purified CD56bright NK cells isolated from resting human peripheral blood failed to demonstrate any significant enhancement of proliferation when incubated in the presence of medium plus SF. It has been shown previously that 10 pM of IL-2 selectively saturates the high affinity IL-2R, constitutively expressed on the CD56bright subset of NK cells and induces a significant proliferative response (2, 3), as evidenced by the 10-fold increase in [3H]thymidine incorporation over baseline seen in Fig. 3 A. The simultaneous addition of ~7 nM SF to cultures containing low (10 pM or ~2.3 U/ml) or high (1 nM or ~230 U/ml) concentrations of IL-2 did not enhance the LAK activity mediated via the high or intermediate affinity IL-2R, respectively (data not shown).

**Figure 1.** Flow cytometric analysis of unsorted PBL from a representative normal donor showing the selective expression of c-kit receptor on the CD56bright subset of human NK cells. (A and B) Analysis of 10,000 PBL, indicating the relative percentage of CD56bright cells. Arrow (B) indicates CD56bright cells in PBL coexpressing c-kit receptor (i.e., ~0.77%), whereas the isotype control (A, quadrant 2) indicates only 0.06% reactivity. In contrast, for the CD56- and CD56dim cells, there is no difference between the isotype control binding (3.89%, A, quadrant 3), and the c-kit receptor binding (3.47%, B, quadrant 3). (C and D) The FI-2 (PE) amplifier gain of the FACScan® is adjusted to trigger selectively on the CD56bright cells while analyzing ~10⁶ unsorted PBL which were simultaneously stained with anti-CD56-PE and either isotype control mAb-FITC (C, quadrant 2) or anti-c-kit receptor-FITC (D, quadrant 2). 3,000 CD56bright cells were collected. (D) 56.1% of these cells coexpress the c-kit receptor; (C) only 3.37% react with the isotype control mAb, giving a net CD56bright c-kit+ of 53%.

**Figure 2.** Functional characterization of the CD56bright c-kit+ cell. CD56bright c-kit+ (•) and CD56bright c-kit- (○) cells were sorted from fresh PBL and either placed immediately into a 4-h cytotoxicity assay with 51Cr-labeled K562 target cells (NK activity), or cultured overnight in the presence of 10 nM IL-2 and then placed into a 4-h cytotoxicity assay with the 51Cr-labeled COLO 205 target cells (LAK activity). E/T ratio was 10:1. Results are the mean ± SE for triplicate wells.
At 10 pM IL-2, the enhancing effect of SF on proliferation of the CD56\textsuperscript{bright} population was shown to be dose dependent, with significant increases in IL-2-induced proliferation consistently being seen at SF concentrations between 70 and 100 pM (data not shown), and a two- to threefold increase in proliferation at SF concentrations between 0.7 and 7 nM (Fig. 3 A). A peak effect was reached at values between 20 and 27 nM (data not shown). The effect of SF was also shown to be time dependent. Cells cultured for 96 h after the simultaneous addition of SF and IL-2 demonstrated a proliferative response that was 40% greater than cells cultured in IL-2 for 24 h before the addition of SF and for cells cultured in SF for 24 h before the addition of IL-2. In addition, CD56\textsuperscript{bright} NK cells cultured simultaneously in SF and IL-2 proliferated in vitro for up to 6 d beyond that seen with IL-2 alone (data not shown).

The addition of SF to CD56\textsuperscript{bright} NK cells cultured at increasing concentrations of IL-2 resulted in a distinct shift of the IL-2 proliferative dose–response curve (Fig. 3 B). In the absence of SF, CD56\textsuperscript{bright} NK cells required 25 pM IL-2 to achieve 50% of the maximal proliferative response, whereas in the presence of ~7 nM SF, CD56\textsuperscript{bright} NK cells achieved a comparable response with only 3 pM IL-2. Importantly, SF induced this synergistic proliferative response only at those concentrations of IL-2 that saturate the high affinity IL-2R (i.e., 1–100 pM) (16). CD56\textsuperscript{bright} cells cultured in the presence of SF and higher concentrations of IL-2 (i.e., 1–10 pM), which saturate both the high and the intermediate affinity IL-2R expressed on these cells, did not show further increases
Discussion

In the present study, we report that the CD56bright subset of human NK cells constitutively expresses functional receptors encoded by the c-kit protooncogene. Whereas the expression of the c-kit receptor has previously been found on a broad range of hematopoietic and nonhematopoietic cell types (14), expression on resting human lymphocytes has not previously been reported. By flow cytometric analyses, c-kit receptor expression on lymphocytes appears to be restricted to the CD56bright NK subset. The studies demonstrating significant NK and LAK activity within the CD56bright c-kit+ fraction provide evidence that this population fulfills the functional definition of NK cells. The CD56bright NK subset represents <2% of human PBL, and also appears to be unique in its constitutive expression of the high affinity IL-2R (2, 3). Given the synergy of SF with other ligands of the hematopoietin receptor superfamily (11, 12), we investigated whether the SF-c-kit receptor interaction would enhance the functional responses of the CD56bright NK subset mediated via the IL-2R.

The results presented here demonstrate that SF significantly enhances the IL-2-induced proliferative response of the CD56bright NK subset, and does so in a dose-dependent fashion. Whereas SF alone has no effect on proliferation, it causes a pronounced shift in the IL-2 dose–response curve at concentrations of IL-2 that selectively saturate the high affinity IL-2R (i.e., 1–100 pM). CD56bright NK cells cultured in the presence of ~7 nM SF required approximately eightfold less IL-2 to achieve 50% of their maximal proliferative response. In addition, the inhibition of IL-2 binding to the high affinity IL-2R with anti-Tac mAb, which results in a profound reduction in IL-2–induced proliferation, proportionally reduced the proliferation achieved with SF and IL-2. Together, these data strongly suggest that signal transduction via the IL-2–high affinity IL-2R interaction is a prerequisite for the enhanced proliferative effect mediated by SF.

The fact that SF did not augment a proliferative response mediated via the intermediate affinity IL-2R at high concentrations of IL-2 is not surprising, since the intermediate affinity IL-2R, expressed on CD56dim or CD56bright NK cells does not transduce a significant proliferative signal when fully saturated by IL-2 (2). However, SF did not augment the NK cytolytic response which is enhanced on all NK cells after activation of the intermediate affinity IL-2R by IL-2 (2). SF may therefore potentiate an intracellular signal that is specific for IL-2–induced proliferation and not IL-2–enhanced cytoxicity.

The expression of the c-kit receptor is most abundant on the early hematopoietic progenitor populations and declines with terminal myeloid and erythroid differentiation (7). Nagler et al. (17) performed a phenotypic and functional analysis of CD56bright (CD16+) and CD56dim (CD16−) human NK cells, and proposed that the CD56bright NK subset is less differentiated than the CD56dim subset. The unique expression of the c-kit receptor on the CD56bright NK subset would lend further support to this proposal, and may help to explain why the CD56bright c-kit+ fraction displays less NK and LAK activity than the CD56bright c-kit− fraction. The proliferation studies performed in the presence of the anti-c-kit receptor mAb provide strong evidence that the potentiating effect of SF on CD56bright NK cells is indeed mediated via the c-kit receptor. In vitro concentrations of SF which approximate those found in vivo (15) were found to significantly augment the IL-2–induced proliferative response of CD56bright NK cells when compared with that seen with IL-2 alone. SF may therefore have physiologic relevance for the CD56bright NK functional response in vivo when, during viral infection, soluble IL-2 is produced by activated T cells. Recent work by Miller et al. (18) suggests that IL-2 and stromal cell factors such as SF are required for NK cell development from CD34+ “DR−” progenitor populations in vitro. The constitutive expression of the c-kit receptor on this NK
subset may therefore also serve to promote intimate contact with marrow stromal cells expressing the active transmembrane form of SF during its maturation in vivo.

In a recent clinical trial, we have demonstrated that the CD56high subset of human NK cells can undergo a profound selective expansion in vivo during a prolonged continuous infusion of low dose IL-2. Serum concentrations of IL-2 during these infusions ranged between 10 and 200 pM, optimal for saturation of the high affinity IL-2R (19). The results of our in vitro study reported here suggest that the presence of SF in normal human serum may be an important component of this selective immune modulation in vivo, and that the concomitant administration of exogenous SF might produce a more rapid expansion of this NK cell subset at a significantly lower concentration of IL-2. Further elucidation of the mechanisms involved in this potentiating effect, as well as other functional consequences of SF on human NK cells, should lend additional insights into the role(s) of this growth factor in NK cell development and the host's normal immune response, and may provide important information for the successful design of future clinical trials.

We thank Mr. David Sheedy for cell sorting. We thank Drs. John Dick, Sharon Evans, C. J. Eaves, Kendall Smith, Carleton Stewart, and Thomas Tomasi for providing insightful discussions.

This work was supported by National Institutes of Health grant CA-01572. M. A. Caligiuri is also supported by an award from the Dr. Louis Sklarow Memorial Fund, an Institutional Research Grant from the American Cancer Society, and the Coleman Leukemia Research Fund.

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Received for publication 2 March 1993 and in revised form 7 May 1993.

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